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DEVELOPMENT OF METHODS FOR CARRIER-MEDIATED TARGETED DELIVERY OF ANTIVIRAL COMPOUNDS USING MONOCLONAL ANTIBODIES

ANNUAL REPORT

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Synthesis and biological evaluation of antiviral drug (ribavirin)-monoclonal antibody conjugates for treatment of Pichinde virus infections.

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SRI International and Utah State University are developing methodology to assess the effectiveness of antiviral drug-monoclonal antibody conjugate therapy for the treatment of viral infections. Specifically, monoclonal antibodies to Pichinde virus surface antigens will be prepared and conjugated to the antiviral drug ribavirin, and the resulting conjugates screened for antiviral activity both <u>in vitro</u> and in animal model systems. Keywords; antiviral agents; antibodies; clones; chemotherapy.

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RESEARCH SUMMARY

Research was conducted to: 1) introduce tether groups onto ribavirin that would permit the attachment of this drug to monoclonal antibodies, 2) develop *in vitro* and *in vivo* Pichinde virus models for use as test systems for evaluating the targeted delivery of antiviral compounds using monoclonal antibodies, and 3) derive and produce monoclonal antibodies against Pichinde virus-coded antigens expressed on the surface of Pichinde virus-infected cells.

1. Synthesis of Ribavirin Derivatives Having Tether Group Functionality.

The 5'-hemisuccinate of ribavirin and the 2',3'-ketals of ribavirin with levulinic acid and 4-acetylbutyric acid and their corresponding sodium salts were prepared, characterized, and submitted for biological screening against Pichinde virus. Work was completed on the synthesis of [1'-^{3}H]ribavirin, which will be used to determine loadings of the drug on the ribavirin-monoclonal antibody conjugates.

2. *In Vitro* Culture of Pichinde Virus.

Cell culture pools of two strains of Pichinde virus, CoAn3739 and An4763, were produced in Vero 76 cells. Initially, conditions for the growth of Pichinde virus in cell culture were determined, and then both strains of the virus were twice plaque-purified. Cell culture pools of Pichinde virus were prepared from the plaque-purified isolates. These pools were used for the production of antigens, as inocula for antibody screening in cell culture, and for the development of the *in vitro* and *in vivo* Pichinde virus models to be used for antiviral testing.

3. Production of Large Pools of Pichinde Virus.

Large pools of Pichinde virus were prepared for use as antigens in the characterization of monoclonal antibodies. Pichinde virus strains CoAn3739 and An4763 were grown in roller bottle cultures of Vero 76 cells. The resulting CoAn3739 virus pool had a titer of 3×10^7 fluorescent cell forming units/ml, and the An4763 virus pool had a titer of 4×10^6 fluorescent cell forming units/ml.

4. Development of Pichinde Virus *In Vitro* Models.

Attempts were made to produce an acceptable cytopathic effect (CPE) in cells using the pools of Pichinde virus. Twelve different

cell lines were studied for their sensitivity to the virus. A slight degree (1+ with 4+ being maximum) of CPE was seen only in African Green Monkey Kidney (Vero 76) cells, and this did not develop until 12 days after the initial exposure of the cells to virus. The inclusion of trypsin with the cells—a method which enhances CPE induced by other viruses--was not effective with Pichinde virus. Varying the concentration of fetal bovine serum (FBS) or including DEAE in the culture medium was also not effective. Plaques were readily produced in Vero 76 cells with both strains of Pichinde virus using a 1% agarose overlay. These plaques were seen in four days when they were enhanced visually with neutral red or crystal violet dye. Preliminary work with an immunofluorescence assay that detects Pichinde virus-infected cells indicates that the immunofluorescence assay might have potential for the <u>in vitro</u> evaluation of antiviral compounds against Pichinde virus.

5. <u>In Vitro Antiviral Assay of Compounds Prepared by SRI International.</u>

Three compounds synthesized by SRI International, and ribavirin, a known antiviral compound were evaluated for <u>in vitro</u> antiviral activity. The three newly synthesized compounds were 1-[5-(carboxy-propionyl)-β-<u>D</u>-ribofuranosyl]-1,2,4-triazole-3-carboxamide (SRI-7422-52), 1-[2,3-<u>O</u>-(1-carboxy-3-butylidene)-β-<u>D</u>-ribofuranosyl]-1,2,4-triazole-3-carboxamide sodium salt (SRI-7422-80), and

1-[2,3-O-(1-carboxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide sodium salt (SRI-7422-82). Ribavirin and SRI-7422-52 were found to be highly active against Punta Toro virus using CPE inhibition as the parameter and against Pichinde Virus using both CPE inhibition and plaque reduction as parameters. Although it used much more test compound, the plaque reduction test was considered the most acceptable for the Pichinde virus.

6. Development of In Vivo Model for Pichinde Virus in MHA Hamsters.

The twice plaque-purified pool of An4763 strain of Pichinde virus was titered in young adult MHA strain hamsters. Subcutaneous injection of the An4763 strain was capable of producing 100% lethality in approximately 12 days, which was associated with severe hemorrhagic disease.

7. Overview of Development of Methodology for the Derivation and Production of Monoclonal Antibodies Against Pichinde Virus-Specified Cell Surface Antigens.

An overview of steps in the derivation and production of monoclonal antibodies against Pichinde virus antigens was developed as a guide for the monoclonal antibody production phases of this project. The overview and accompanying flow chart describes the sequence, as

well as the rationale, for the various steps. Those experiments or steps that have been completed in the first year are listed in the following sections of this summary and are described in detail in the subsequent sections of this report.

8. Preparation of Immunogens and Hyperimmunization of Spleen Donor Mice.

Suckling mouse brain pools of Pichinde virus were used as immunogens in spleen donor mice. Seperate immunogen pools were prepared for each of the two strains of mice used as spleen donors. Suckling RBF/DnJ mice and BALB/c mice were inoculated intracerebrally with twice plaque-purified An4763 Pichinde virus. From each pool of suckling mouse brains, a 10% homogenate in PBS was prepared. The RBF/DnJ suckling mouse brain homogenate was used to immunize eight-week-old RBF/DnJ mice; the BALB/c suckling mouse brain homogenate was used to immunize eight-week-old BALB/c mice. The RBF/DnJ spleen donor mice achieved serum antibody titers in excess of 1:6,400 as measured by indirect immunofluorescence, whereas the BALB/c serum antibody titers were between 1:100 and 1:200. The RBF/DnJ suckling mice inoculated by intracerebral injection with Pichinde virus were severely affected and might serve as a useful model for arenavirus encephalitis.

9. Production of Anti-Pichinde Virus Antisera.

Both hyperimmune mouse sera and hyperimmune ascites fluids were produced in mice hyperimmunized by intramuscular injections with Pichinde virus. These antisera were used as test reagents and as positive controls in the development of anti-Pichinde virus antibody detection procedures. The hyperimmune mouse sera pool had an anti-Pichinde virus titer of 1:640 as measured by indirect immunofluorescence, whereas the titer of the hyperimmune ascites fluids was 1:200.

10. Development of Fluorescent Antibody Assay for Pichinde Virus or Antibody to Pichinde Virus.

Immunofluorescence procedures for assaying either Pichinde virus or antibody to Pichinde virus were established using acetone-fixed Pichinde virus-infected Vero 76 cells as targets for fluorescent antibody staining using either direct or indirect fluorescent antibody procedures. Primary hyperimmune antisera were prepared in mice, and several fluorescein-labeled anti-mouse antisera were purchased and evaluated.

11. <u>Development of Detection Procaduras Specific for Antibodies toward Cell Surfeca-Associated Virus Antigens end Their Applicetion to Screaning Hybridomas for Antibody to Pichinda Virus.</u>

When infactad calls are to be used es tha targets in immunofluorescence assays for call surfeca-essocietad virel antigens, the calls must not be made permeable as thay era in the casa whan they ara fixed with acatone or othar membrane-dissolving fixativas. After axamining savarel nonpermeebilizing fixatives, we found that calls fixed with 4% formaldehyde became firmly attached to the plastic cell culture plate and, under certain conditions, were suitebly impermeeble to entibodias. An indirect immunofluorescence essey utilizing formeldehyde-fixed target calls was davelopad as e screening procadura for spacific antibodies to call surfeca-essocietad viral antigens. A result of thesa studias was the davalopment of e two-staga procadure for screaning hybridomas, in which both steges utilized indirect immunofluorescence asseys. The first stage eliminetad tha majority of the inappropriete antibodies. The more rigorous second stege was designed to detect entibodies spacific for cell surface-associated Pichinde virus entigans, to ellow tha elimination of antibodias raecting with Pichinde virus antigens not exprassed on the cell surfece, and to evoid the selection of antibodies reacting with Vero cell antigens.

12. Culturing the FOX-NY Cell Line.

The FOX-NY cell line is used as the myeloma cell line in these hybridization procedures. After the FOX-NY cell line was brought into our laboratory, its growth characteristics were determined in the various media that were to be used for the maintenance, fusion, cloning, and freezing of the cell line and the derived hybridomas. We measured the effects of several commercially available media supplements as well as antibiotics on the growth of FOX-NY cells. None of the media supplements demonstrated significant positive effects on FOX-NY cell growth. When RPMI-1640 medium supplemented with 15% FBS was used as the growth medium, the cells maintained a healthy morphology and demonstrated logarithmic growth over cell densities between 1×10^5 and 2×10^6 cells/ml with a doubling time of 18 to 24 hours. Conditioned medium--RPMI-1640 medium removed from a log-phase culture of FOX-NY cells--was found to be superior to fresh RPMI-1640 for cloning the FOX-NY cell line.

13. Development of a Procedure for Freezing Cells.

A low-technology procedure for freezing cells that did not require specialized cell freezing equipment was developed and determined to be suitable for the FOX-NY cell line. FOX-NY cells in RPMI-1640 medium supplemented with 20% FBS and 10% dimethyl sulfoxide were placed in a styrofoam container and frozen in a conventional -80°C laboratory freezer. After storage at -80°C for 30 days, FOX-NY

cultures frozen by this procedure were found to yield 70% viable cells within 24 hours of recovery. These thawed cultures attained exponential-phase growth within 48 hours. For longer term storage, cells are being stored in the vapor phase of a liquid nitrogen freezer.

14. Fusion Technique for the Generation of Hybridomas.

A fusion technique was established and used for generating hybridomas secreting anti-Pichinde virus antibody. The procedure utilizes the FOX-NY cells, which are fused to spleen cells of hyperimmunized RBF/DnJ strain mice using polyethylene glycol. The first two fusions did not yield viable hybridomas. It was determined that the polyethylene glycol used in the procedure was cytotoxic. Several different types of polyethylene glycol were tested and one of the least cytotoxic was used in a subsequent fusion. Viable hybridomas from that fusion are now being cultured.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

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STATEMENT OF PROBLEM UNDER STUDY

Under USAMRIID Contract No. DAMD17-86-C-6120, SRI International and Utah State University are developing methodology to assess the effectiveness of antiviral drug-monoclonal antibody conjugate therapy for the treatment of viral infections. Specifically, we are undertaking the preparation of monoclonal antibodies directed against viral antigens expressed on the surface of Pichinde virus, the conjugation of the antiviral drug ribavirin to these monoclonal antibodies, and the biological assessment of the antiviral activity of these conjugates compared with that of free drug in virally infected cells in culture and in hamsters.

BACKGROUND

Approximately 60% of human illnesses are caused by viral infections.[1] In particular, military personnel may be exposed to many different virulent pathogens when deployed abroad. The spread of these pathogens is exacerbated by inadequate sanitation, poor health conditions, and crowded living and working conditions. The arenaviruses (Junin, Lassa, and Machupa) cause hemorrhagic fevers that have high mortality rates in humans. These diseases are characterized by high fever, leucopenia, gastrointestinal hemorrhagic manifestations, shock, and, in some instances, a neurologic syndrome.[2] The duration of illnesses is generally two to three weeks, and complete recovery requires long convalescence times. The mechanism of pathogenesis for these viruses in humans appears to be direct damage to the cells. The viruses cause extensive capillary damage (either directly or indirectly), which has been proposed as the causative factor of the organ damage resulting from these diseases.[3]

Because vaccination therapy or drug therapy for many of the infections caused by members of the Arenaviridae is not available or effective, new methods of treatment must be developed. In addition, in those cases where antiviral drugs have been shown to inhibit the virus *in vitro*, effective therapy *in vivo* may require the

administration of such high drug doses that toxic side effects occur. Therefore, by targeting the delivery of antiviral drugs to the virus or the virally infected cell, lower concentrations of drugs could be administered, thereby reducing their toxic side effects.

RATIONALE

Targeted drug therapy using antiviral drug-monoclonal antibody conjugates has potential value for the treatment of viral infections. Monoclonal antibodies (MAbs) to specific antigens expressed on the viral surface or on the surface of virally infected cells would specifically deliver the conjugated drug to the virus or the infected cell. Therefore, drug efficacy would be enhanced and systemic side effects would be reduced relative to standard treatment modalities using unbound drugs.

The drug-monoclonal antibody method of drug delivery is currently being investigated as a new therapeutic approach for the treatment of cancer.[4-19] Monoclonal antibodies that are specific for antigenic determinants on tumor cells have been developed.[5,7] The chemotherapeutic drugs doxorubicin,[8-10] daunomycin,[11-13] and methotrexate,[14] the toxin ricin.[15,16] other toxins,[17] and the protein A chain of ricin[18-19] have been covalently linked to antibodies against antigens on tumor cell lines. The resultant conjugates have been screened in both <u>in vitro</u> and animal model systems. Conjugation of both drugs and toxins did not interfere with the binding of antibodies to their antigenic determinants. The conjugates had increased specific cytotoxicity to cancer cells in culture compared with control cells lacking the

antigens.[11,13] Most important, the conjugates prolonged the survival times of animals infected with the cancer cells over those of infected animals treated with the unbound drugs or antibodies alone or with combinations of free drugs with the antibodies.[12]

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TASK REPORTS

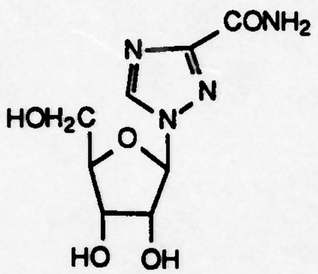
This annual progress report describes research accomplished in the first ten months of the contract (September 30, 1986 to February 15, 1987) of USAMRIID Contract No. DAMD17-86-C-6120. Because of the extensiveness and diversity of this multidisciplinary project, the following report has been subdivided into particular task reports, which are presented in the order set forth in the Research Summary.

I. Synthesis of Ribavirin Derivatives Having Tether Group Functionality

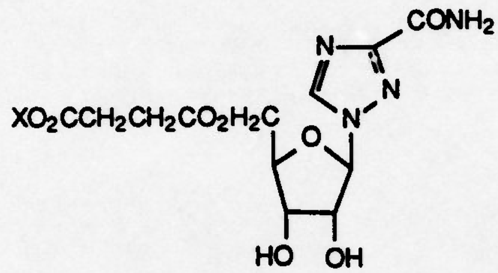
A. Introduction

Because of its high antiviral potency, ribavirin (1, Chart I-1)[1,2] was selected as the drug of choice to assess the potential of targeted drug delivery using monoclonal antibodies. The 5'- and the 2',3'-positions on the ribofuranose ring of 1 were selected to introduce the tether functionality by which the drug would be attached to the MAb. The tether functionality had to be so designed that the covalent linkage of ribavirin to the MAb would be sufficiently stable to enable the conjugate to srrive intact at the surface of the virally

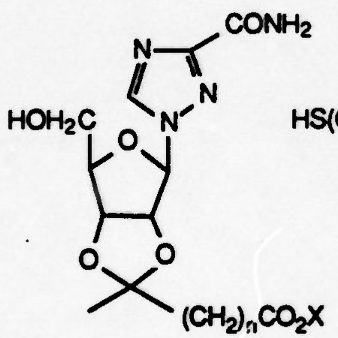
Chart I-1. Ribavirin and its Functionalized Derivatives



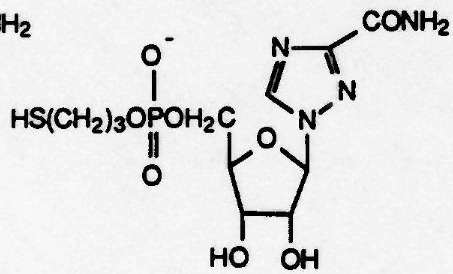
1



2 X = H
3 X = Na



4 n = 2, X = H
5 n = 2, X = Na
6 n = 3, X = H
7 n = 3, X = Na



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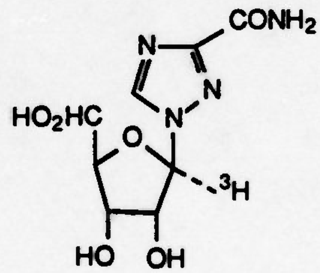

9

infected cell. Once at the cell surface, drug release from the conjugate could occur by one of two pathways: 1) chemical or enzymic hydrolysis of the conjugate at the cell surface to afford a high local concentration of the drug, which could then enter the cell by its usual active or passive transport processes or 2) invagination of the conjugate by the cell followed by lysozomal cleavage of the conjugate to release the free drug within the cell. Ester, ketal, and phosphate groups were initially proposed for linking ribavirin to MAbs. The initial synthetic targets were the 5'-hemisuccinate ester 2 of ribavirin, the 2',3'-ketal of ribavirin with levulinic acid (4), the 2',3'-ketal of ribavirin with 4-acetylbutyric acid (6) and the 5'-phosphate diester of ribavirin with 3-mercaptopropanol (8). Because of the low storage stability of hygroscopic hemisuccinate 2 and the ketals 4 and 6, the corresponding sodium salts 3, 5, and 7 were also targeted for synthesis.

To accurately assess the biological activity of the drug-MAb conjugates, it is necessary to determine the number of drug molecules attached to each antibody. Because ribavirin has a very weak ultraviolet chromophore, conventional loading determinations using spectroscopic absorption were not possible in the presence of protein and so we decided to determine drug loading using radiolabeled ribavirin. Therefore, the synthesis of [1-^{3}H]ribavirin (9) was undertaken.

B. Experimental Methods

General Procedures and Instrumentation. When required, reactions and purifications were conducted with deoxygenated solvents and under inert gas (argon) and subdued light. Solvents were dried or distilled before use. Melting points were uncorrected. TLC analyses were performed on Analtech silica gel analytical plates, using detection by UV or anisaldehyde spray (0.5 ml of anisaldehyde in 0.5 ml of concentrated sulfuric acid added to 9 ml of 95% ethanol containing a few drops of acetic acid, with the sprayed plate heated to 100-110°C for 20 to 30 min). Silica gel 60 (E. Merck No. 9385) for flash column chromatography was obtained from Brinkman. A Pharmacia Ultrarac fraction collector equipped with a UV detector (280 nm) and a peristaltic pump was used to collect column fractions where required. Lyophilizations were performed using a Labconoco lyophilizer. HPLC analyses were performed on a Spectra Physics SP8100 instrument fitted with a Waters radial compression C18 Nova-Pak reverse-phase column with a SP8440 UV/vis detector set at 254 nm and a SP4200 computing integrator. Injection samples (20 μl) were prepared at a compound concentration of 1 mg/ml. Elution was performed using the following linear gradient system: 1) 98% 0.1 M sodium acetate/2% methanol (0 to 5 min); and 2) 98-80% 0.1 M sodium acetate/2-20% methanol (5 to 16 min) at a flow rate of 2.0 ml/min. IR spectra were recorded with a Perkin-Elmer 710B infrared spectrometer. NMR spectra were obtained with a JEOL FX90Q or Varian 400 MHz spectrometer, using tetramethylsilane as an internal standard (δ 0). UV spectra were taken on a Perkin-Elmer 575

spectrophotometer. Optical rotations were performed on a Perkin-Elmer 141 Polarimeter. Elemental analyses were conducted by MicAnal, Tucson, Arizona. Mass spectral analyses were performed by Dr. David Thomas, SRI International, using a Ribermag Model R10-10C mass spectrometer. Samples were introduced using the desorption probe for both electron impact and chemical ionization studies. The number in parentheses appearing at the end of each experimental procedure refers to the SRI notebook number and page number for the start of that experiment. Only procedures for those synthetic steps in the successful routes to the target compounds are described.

1-Cyanoformimidic Acid Hydrazide (12). A literature procedure[3] was followed. Cyanogen gas (10) (Matheson) was bubbled into 450 ml of dioxane at room temperature until 78.0 g (1.5 mol) of the gas had been dissolved (2 h). The solution was cooled to 5°C. A solution of 50.0 g (49.5 ml, 1.56 mol) of 95% hydrazine (11) (Eastman) in 165 ml of 10:1 dioxane/methanol was added through a dropping funnel to the magnetically stirred cyanogen solution at 5°C over a period of 1.75 h. The solution became yellow, and a white precipitate formed. At the end of the addition period, the reaction mixture was red and efficient stirring became very difficult. After the addition was complete, the reaction mixture was allowed to stand for 2.5 h at 5°C and was then filtered (Whatman No. 1 paper). The orange crystals were washed once with dioxane and then dissolved in 300 ml of hot isopropyl alcohol. The hot alcohol solution was treated with 1 g of Norit, filtered, and cooled to 50°C. Hexane was added to the solution until cloudiness

persisted. On cooling to room temperature, crystals formed. They were collected (coarse glass-frit filter) and dried (0.025 mm Hg, 24 h) to give 55 g (44% yield) of 12, mp 79-81°C (lit[3]: 82-86°C); IR (KBr) 2800-3450, 2225, 1630, 1350, 1105-1160, 1090, 1045 cm^{-1}. This material was used immediately in the following reaction. Storage of a sample of 12 for eight months at room temperature resulted in decomposition as evidenced by darkening.

(6898-46)

3-Cyano-1,2,4-triazole (14). A literature procedure[2] was used. To a stirred solution of 49.3 g (586 mmol) of 1-cyanoformimidic acid hydrazide (12) in 293 ml of triethyl orthoformate (13) at 0°C was added 15.6 ml of dioxane that had been saturated at room temperature with hydrogen chloride gas. The solution turned red and a white precipitate formed after 1 h. After 5 h at 0°C and 15 h at room temperature, the now yellow solution was concentrated to dryness at 50°C. The residue was taken up in 1 L of ether. This solution was washed with 50 ml of water, filtered, dried (magnesium sulfate), and concentrated to a solid, which was crystallized (200 ml of ethyl acetate and 300 ml of benzene concentrated to a volume of 200 ml) to give 17.0 g of 14 as white crystals, mp 183-184°C (lit[2]: 185-187°C); 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 8.93 (s, HetH). The solid recovered from filtration of the ether extract was also recrystallized (ethyl acetate/benzene) to give 10.6 g of 14, mp 183-185°C. The total yield was 27.6 g (50%).

(6898-48)

1,2,3,5-tetra-O-Acetyl-β-D-ribofuranose (15). According to a literature procedure,[4] 1.00 g (6.67 mmol) of D-ribose (47) was dissolved with stirring in 16 ml of methanol under argon. The clear solution was cooled to 0°C before 80 μl of concentrated sulfuric acid was added dropwise. After being stirred at 4°C for 12 to 14 h, the reaction mixture was neutralized with 3 ml of pyridine and concentrated at reduced pressure (15 mm Hg) to give 1.1 g of the α,β-methylriboside as a thick, light-brown oil. The oil was dissolved in 3.0 ml of acetic acid, and then 4.0 ml (36 mmol) of acetic anhydride was added. After cooling to 0°C under argon, the solution was treated with 120 μl of concentrated sulfuric acid and then allowed to come to ambient temperature over a period of 1 h. The reaction mixture was then cooled to 0°C, treated with another 200-μl portion of sulfuric acid, and allowed to return to room temperature over a period of 2 h. Next, 1.20 g (14.6 mmol) of anhydrous sodium acetate and 30 ml of ethanol was added. This mixture was concentrated to dryness below 50°C and submitted to one of the two purification methods described below.

Purification A. The residue was reconcentrated from three 35-ml portions of ethanol and then dissolved in 25 ml of chloroform. The chloroform solution was washed with 20 ml of water, dried (magnesium sulfate), and concentrated to a viscous brown oil, which was dried (0.03 mm Hg). The crude tetra-acetate was dissolved in 5 ml of hot ethanol. The solution was cooled to 0°C for 1 h and to -20°C for

1.5 h. The mother liquors were removed, and the crystals were recrystallized at -20°C from 1.5 ml of ethanol and dried (0.025 mm Hg, room temperature) to afford 541 mg (26% yield) of tetra-acetate 15 as a white crystalline powder, mp 78-79°C (lit[5]: 79-81°C); TLC (5% methanol/chloroform, anisaldehyde detection) R_f 0.64 (authentic 15).

Purification B. The crude tetra-acetate was dissolved in 3 ml of chloroform and washed with four 5-ml portions of water, dried (magnesium sulfate), and concentrated at reduced pressure. The oily residue was dissolved in 7 ml of ethanol. The ethanol solution was concentrated to a volume of 4 ml, seeded, allowed to stand at room temperature for 1 h, and then cooled to 0°C for 1 h. Filtration with a rinse of two 5-ml portions of -20°C ethanol, followed by drying, gave 520 mg (24% yield) of 15.

(7421-48; 7422-84)

3-Cyano-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1,2,4-triazole (17). Following a literature procedure,[2] a mixture of 50.0 g (157 mmol) of 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (15) and 14.76 (157 mmol) of 3-cyano-1,2,4-triazole (14) was magnetically stirred under argon in a 150-160°C oil bath. After the reaction mixture melted, 160 mg (0.14 mmol) of bis(4-nitrophenyl) phosphate (16) was added. The reaction vessel was evacuated (0.025 mm Hg) and the acetic acid that formed was collected by distillation into a cold trap cooled to -78°C. Heating was continued at 150-160°C (external temperature) for 15 min. The brown reaction mixture was cooled to room temperature, at which

point it solidified. The solid was dissolved in 200 ml of chloroform, treated with Norit, and filtered. The Norit treatments were repeated until an almost colorless filtrate was obtained. The chloroform was removed by concentration at reduced pressure to give a viscous oil to which 600 ml of ether was added. This mixture was heated on the steam bath to dissolve the oil. Concentration to 400 ml and cooling afforded 24.5 g of white crystals, mp 95-97°C (lit[2]: 96-97°C). Concentration of the mother liquors to 125 ml and cooling afforded 9.3 g of 17, mp 96-98°C. Further cooling produced another 9.4 g of 17, mp 96-97°C. The total yield of 17 was 43.2 g (78%); IR (chloroform) 2875, 1690, 1590, 1490, 1405, 1360, 1080, 1020, 990 cm^{-1}; 90 MHz ^{1}H NMR (dimethyl sulfoxide-d_6) δ 1.99 (s, 3 H, $COCH_3$), 2.07 (s, 3 H, $COCH_3$). 2.09 (s, 3 H, $COCH_3$), 4.10 (m, 1 H, 4'-CH), 4.35 (m, 2 H, 5'-CH_2), 5.40-5.70 (m, 2 H, 2',3'-CH), 6.40 (d, J = 2.6 Hz, 1 H, 1'-CH), 9.10 (s, 1 H, 5-HetH).

(7421-50; 7422-72)

1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin, 1). According to a literature[2] procedure, 36.7 g (102 mmol) of 3-cyano-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1,2,4-triazole (17) was dissolved in 260 ml of concentrated ammonium hydroxide by warming on the steam bath. Warming was continued for 1 h at which time TLC (15:10:2 chloroform/methanol/water) showed one spot (R_f 0.76) that cospotted with a sample of ribavirin supplied by Dr. Robert Sidwell, Departments of Biology and Animal, Dairy, and Veterinary Sciences, Utah State University. The reaction mixture was cooled to room temperature and

concentrated at reduced pressure below 50°C to give a syrup, which was dissolved in 600 ml of hot anhydrous ethanol. After decolorizing with activated charcoal, the solution was reduced in volume to 200 ml to remove traces of water. Crystallization was induced by seeding at room temperature and overnight cooling at 4°C to give 23.0 g (92% yield) of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (1) as a white microcrystalline solid, mp 171-173°C (lit[1,2]: 174-176°C); HPLC t_R 2.63 min (100%); 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 3.57 (m, 2 H, 5'-CH_2), 3.94 (m, 1 H, 4'-CH), 4.13 and 4.34 (2 m, 2 H, 2',3'-CH), 4.90 (broad s, 1 H, OH), 5.20 (broad s, 1 H, OH), 5.58 (broad s, 1 H, OH), 5.81 (broad d, J = 4 Hz, 1 H, 1'-CH), 7.60 (broad s, 1 H, NH), 7.82 (broad s, 1 H, NH), 8.86 (s, 1 H, 5-HetH); ^{13}C NMR (dimethyl sulfoxide-d_6) 71.3, 80.0, 84.5, 95.5, 101.8, 155.0. 167.3, 170.4 ppm.

(6898-56, 7422-74)

2-Trimethylsilylethanol (27). A literature procedure[6] was followed. Trimethylsilylmagnesium chloride was formed by reaction of 20.0 g (160 mmol) of α-(chloromethyl)trimethylsilane (25) and 3.96 g (163 mmol) of magnesium turnings in 90 ml of anhydrous ether with intermittent ice-bath cooling to maintain the reaction at a gentle reflux after initiation by gentle heating. When all the magnesium had reacted, 5.0 g (180 mmol) of paraformaldehyde (26) was added, and the mixture was heated at a vigorous reflux for 15 min and a gentle reflux for 2 h. After cooling to room temperature, the reaction mixture was diluted with ether and treated with saturated ammonium chloride. The aqueous layer was extracted twice with ether. The combined ether

fractions were dried (sodium sulfate) and concentrated at reduced pressure to give an oil, which on evaporative distillation (50°C, 14 mm Hg) using a Kügelrohr apparatus afforded 7.56 g (40% yield) of 27 as a colorless oil; ^{1}H NMR (deuteriochloroform) δ 0.00 (s, 9 H, $Si(CH_3)_3$), 0.92 (m, 2 H, CH_2Si), 1.43 (s, 1 H, OH), 3.73 (m, 2 H, CH_2O).

(7422-34)

2'-Trimethylsilylethyl Hemisuccinate (28). To 64 ml of ethyl acetate were added consecutively 7.73 g (77 mmol) of succinic anhydride (18), 7.56 g (64 mmol) of 2-trimethylsilylethanol (27), and 7.77 g (77 mmol) of triethylamine. This mixture was rapidly stirred at room temperature until a clear solution was obtained (1 h). The solution was heated at reflux for 1 h, cooled to room temperature, and concentrated at reduced pressure to an oil, which was diluted with 150 ml of 5% sodium bicarbonate solution and washed with 100 ml of ether. The aqueous layer was acidified to pH 2 with 2 M sulfuric acid, and the oil that separated out was extracted into two 100-ml portions of ether. This ether extract was washed with 100-ml portions of water and saturated brine, dried (sodium sulfate), filtered, and concentrated at reduced pressure to give 9.23 g (85% yield) of 28 as a slightly yellow oil, which was used without further purification in the next step; 90 MHz ^{1}H NMR (deuteriochloroform) δ 0.01 (s, 9 H, CH_3), 0.96 (m, 2 H, CH_2Si), 2.61 (m, 4 H, CH_2CO), 4.17 (m, 2 H, CH_2O), 9.97 (broad s, 1 H, CO_2H).

(7422-36)

t-Butyldimethylsilyl 2'-Trimethylsilylethyl Succinate (30). To a solution of 6.35 g (29.0 mmol) of 2'-trimethylsilylethyl hemisuccinate (28) in 12.7 ml of dimethylformamide containing 4.67 g (31.0 mmol) of t-butyldimethylsilyl chloride (29), cooled in an ice bath, was added 4.04 g (59.4 mmol) of imidazole. The biphasic reaction mixture was rapidly stirred for 24 h under argon to ensure mixing and then was poured into 500 ml of 2.5% sodium bicarbonate solution, which, in turn, was extracted with two 200-ml portions of hexanes. The organic extracts were dried (magnesium sulfate), filtered, and concentrated at reduced pressure to give 9.03 g (94% yield) of 30 as a colorless liquid; IR (film) 2950, 2935, 2900, 2860, 1740, 1725, 1470, 1420, 1370, 1350, 1320, 1260, 1220, 1165, 1070, 1040, 1000, 940, 840, 795, 700 cm^{-1}; 90 MHz ^1H NMR (deuteriochloroform) δ 0.02 (s, 9 H, $Si(CH_3)_3$), 0.24 (s, 6 H, $Si(CH_3)_2$), 0.80-0.95 (m, 2 H, $SiCH_2$), 0.91 (s, 9 H, $C(CH_3)_3$), 2.58 (m, 4 H, CH_2CO), 4.17 (m, 2 H, CH_2O).

(7421-30)

2'-Trimethylsilylethyl Succinyl Chloride (32). A reported procedure[7] was modified. To a solution of 5.00 g (15.0 mmol) of t-butyldimethylsilyl 2'-trimethylsilylethyl succinate (30) in 16 ml of dichloromethane containing 0.20 ml of dimethylformamide, which was cooled in an ice bath, was added dropwise 2.33 g (18.3 mmol) of oxalyl chloride (31) over a period of 5 min. Gas evolved after 0.5 min and continued for several minutes after the end of the addition. Stirring at 0°C was continued for 1 h. The solvent was removed at reduced

pressure (40 mm Hg, 0°C; 0.025 mm Hg, room temperature) giving a colorless, viscous oil, which was evaporatively distilled using a Kügelrohr apparatus to first remove t-butyldimethylsilyl chloride (29) (0.025 mm Hg, < 53°C) and then to collect 1.82 g (51% yield) of 2'-trimethylsilylethyl succinyl chloride (32) (0.025 mm Hg, 60°C) as a colorless oil, which was used without further purification in the next step; IR (film) 2950, 2890, 1800, 1735, 1410, 1390, 1360, 1340, 1300, 1250, 1200, 1175, 1060, 995, 915, 860, 840, 760, 700 cm^{-1}.

(7421-32; 7422-44)

1-{5-[3-(2-Trimethylsilyl)carbethoxypropionyl]-β-D-ribofuranosyl}-1,2,4-triazole-3-carboxamide (2-Trimethylsilylethyl Ester of Ribavirin 5'-Hemisuccinate, 33). A solution of 715 mg (2.93 mmol) of ribavirin (1) in 2.2 ml of N-methylpyrrolidinone was prepared by warming and stirring. To this solution at room temperature was added 830 mg (3.51 mmol) of 2'-trimethylsilylethyl succinyl chloride (32), followed by a 2.3-ml rinse with N-methylpyrrolidinone under argon. The reaction mixture was stirred at room temperature under argon for 15 h. The solvent was removed by evaporative distillation (0.025 mm Hg, < 50°C) using a Kügelrohr apparatus. The residue was purified by flash column chromatography (0 to 10% methanol/chloroform, 1% gradient steps of 250 ml, with 30-ml fractions being collected starting at 3% methanol). Fractions 31 to 50 gave, after concentration at reduced pressure, 930 mg (72% yield) of the 2-trimethylsilylethyl ester of ribavirin 5'-hemisuccinate (33) as a white foam, mp 86-89°C; $[\alpha]_D^{23}$ -4.8° (9.3×10^{-3}, athanol); IR (Nujol mull) 3400, 3340, 1735, 1660,

1625, 1280, 1170, 1150, 1080, 975, 935, 885, 860, 820, 720 cm^{-1}; 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 0.00 (s, 9 H, $Si(CH_3)_3$), 0.90 (m, 2 H, $SiCH_2$), 2.52 (m, 4 H, $(CH_2)_2CO$), 3.95-4.40 (m, 7 H, 2',3',4'-CH, 5'-CH_2, CH_2OCO), 5.43 (broad s, 1 H, OH), 5.69 (broad s, 1 H, OH), 5.86 (d, J = 2 Hz, 1 H, 1'-CH), 7.61 (broad s, 1 H, NH), 7.81 (broad s, 1 H, NH), 8.80 (s, 1 H, 5-HetH).

(7422-46)

<u>1-[5-(3-Carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (Ribavirin 5'-Hemisuccinate, 2).</u>[8] To 1.0 ml of 1 M tetra(n-butyl)ammonium fluoride (1.0 mmol) in tetrahydrofuran (Aldrich) was added 100 mg (0.225 mmol) of the 2-trimethylsilylethyl ester of ribavirin 5'-hemisuccinate (33). The resulting solution was stirred under argon at room temperature for 18 h, at which time TLC (10% methanol/chloroform) indicated only the product 2 (R_f 0.33) and no 33 (R_f 0.77). The solvent was removed at reduced pressure, and the residue was dissolved in 10 ml of ammonium bicarbonate solution (water containing sufficient 5% ammonium bicarbonate to solubilize the solid) and loaded on a DEAE Sephadex A-25 (HCO_3^- form) column (1.5 × 23 cm), which was eluted with 30 ml of water, followed by a linear gradient of 0.0 to 0.5 M ammonium bicarbonate (1-1 volume). The column flow rate was 1.5 ml/min, and fractions of 12-ml volume were collected. The major peak (detected by UV at 280 nm) was eluted in Fractions 12 to 16, which were lyophilized to dryness and then twice lyophilized from 100 ml of water. Lyophilization did not remove all the ammonium bicarbonate according to the ^1H NMR spectrum of the sample.

Therefore, the sample was dissolved in 10 ml of water and chromatographed on a Dowex 50W-X4 (H^+ form) column (2.5 × 18 cm) with water as the eluant (15-ml fractions) at a flow rate of 1.5 ml/min. UV detection indicated that Fractions 9 to 11 contained the major peak. Lyophilization afforded 37 mg (50% yield) of ribavirin 5'-hemisuccinate (2) as an extremely hygroscopic white solid, mp 65-67°C; HPLC t_R 3.60 min; IR (mull) 2500-3500, 1718, 1680, 1290, 1160 cm^{-1}; 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 2.43 (m, 4 H, CH_2CO), 4.00-4.50 (m, 5 H, 2',3',4'-CH, 5'-CH_2), 5.00-5.70 (m, OH), 5.81 (d, J = 2 Hz, 1 H, 1'-CH), 7.55 (broad s, 1 H, NH), 7.75 (broad s, 1 H, NH), 8.90 (s, 1 H, HetH).

(7422-52)

<u>1-[5-(3-Carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (Ribavirin 5'-Hemisuccinate Sodium Salt, 3).</u> A solution of 654 mg (1.46 mmol) of the 2-trimethylsilylethyl ester of ribavirin 5'-hemisuccinate (33) in 6.5 ml of 1 M tetra(n-butyl)-ammonium fluoride (6.5 mmol) in tetrahydrofuran was stirred at room temperature overnight, at which time TLC (10% methanol/chloroform) indicated 3 (R_f 0.27) and no 33 (R_f 0.73) was present. The reaction mixture was concentrated at reduced pressure, and the residue was dissolved in water containing sufficient 0.5 M ammonium bicarbonate to bring about dissolution. The solution was chromatographed on DEAE Sephadex A-25 (30 ml of water, followed by 1000 ml of a 0 to 0.5 M ammonium bicarbonate gradient), with 12-ml fractions being collected. Fractions 12 to 16 were pooled and lyophilized. The residue was

redissolved in water and lyophilized. The residue was dissolved in water and chromatographed four times on a Dowex 50W-X4 (Na^+) column (2.5 × 18 cm) using water as the eluant and collecting 20-ml fractions. The product fractions were pooled and lyophilized to afford 246 mg (46% yield) of sodium salt 3 as a white solid, mp 158-160°C; $[\alpha]_D^{25}$ -5.8° (8.1 × 10^{-3}, water); IR (Nujol mull) 2500-3680, 1730, 1690, 1580, 1295, 1175, 1090, 885, 835 cm^{-1}; ^1H NMR (dimethyl sulfoxide-d_6) δ 2.13 (d, J = 5 Hz, 2 H, CH_2CO_2), 2.32 (d, J = 5 Hz, 2 H, CH_2CO_2), 3.9-4.5 (m, 5 H, 2',3',4'-CH, 5'-CH_2), 5.52 (d, J = 3 Hz, 1 H, 1'-CH), 7.56 (broad s, 1 H, NH), 7.89 (broad s, 1 H, NH), 8.80 (s, 1 H, 5-HetH); ^{13}C NMR (dimethyl sulfoxide-d_6) 40.8, 42.3, 73.7, 80.4, 84.2, 92.0, 101.8, 155.4, 167.4, 170.5, 183.3, 185.9 ppm; MS m/e $[M + (TMS)_4]^+$ 632.

(7422-88)

1-[2,3-O-(1-Carbethoxy-3-butylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (2',3'-Ketal of Ribavirin with Ethyl Levulinate, 37). To a stirred solution of 14.2 g (99 mmol) of ethyl levulinate (35) and 17.8 g (120 mmol) of triethyl orthoformate (13) was added under argon 3.00 g (12.3 mmol) of ribavirin (1). This mixture was cooled in an ice bath while 0.66 ml of 70% perchloric acid was added dropwise with rapid stirring. The reaction mixture was allowed to come to room temperature and then was stirred for 2 h to completely dissolve the ribavirin. After 4 h at room temperature, TLC (10% methanol/chloroform) of the reaction mixture indicated product (R_f 0.54), no ribavirin (R_f 0.07), and unreacted ethyl levulinate (R_f

0.97). The crude reaction mixture was partitioned between 50 ml of chloroform and 50 ml of 5% sodium bicarbonate solution. The chloroform layer was dried (sodium sulfate) and concentrated at reduced pressure to give an oil, which was triturated at 4°C with 100 ml of hexanes for 16 h. The residue from trituration was purified by flash column chromatography (0-10% methanol/chloroform, gradient steps of 2% methanol of 300, 500, 300, and 600 ml, with 25-ml fractions being collected beginning at 6% methanol) to give, after concentration at reduced pressure, 2.80 g (61% yield) of a slightly yellow solid. Crystallization from dichloromethane/ether gave 2.16 g (47% yield) of ketal 37 as a white solid, mp 66-69°C; $[\alpha]_D^{23}$ -39.3° (13.2×10^{-3}, 95% ethanol); IR (Nujol mull) 3300, 3100, 1720, 1680, 1620, 1300, 1180, 890, 860, 720 cm^{-1}; 400 MHz ^1H NMR (deuteriochloroform) δ 1.24 (t, J = Hz, 3 H, $\underline{CH}_3CH_2$), 1.32 (s, 3 H, CH_3C), 2.16 (dd, J = 7 Hz, J = 2 Hz, 2 H, $\underline{CH}_2CH_2CO_2$). 2.48 (t, J = 7 Hz, 2 H, CH_2CO_2), 3.69 (dd, J = 13 Hz, J = 3 Hz, 1 H, 5'-CH_2), 4.13 (q, J = 7 Hz, 2 H, $CH_3\underline{CH}_2$), 4.52 (s, 1 H, 4'-CH), 5.02 (d, J = 6 Hz, 1 H, 3'-CH), 5.22 (dd, J = 6 Hz, J = 2 Hz, 1 H, 2'-CH), 6.08 (d, J = 2 Hz, 1 H, 1'-CH), 6.14 (broad s, 1 H, NH), 7.06 (broad s, 1 H, NH), 8.39 (broad s, 1 H, 5-HetH); ^{13}C NMR (dimethyl sulfoxide-d_6) 24.1, 33.4, 38.0, 43.2, 69.9, 71.2, 91.7, 94.1, 98.4, 102.5, 123.2, 155.2, 167.4, 170.3, 182.6 ppm. Anal. calcd for $C_{15}H_{22}N_4O_7$: C 48.65, H 5.99, N 15.13; found: C 48.35, H 6.16, N 14.91.

(7422-76)

1-[2,3-O-(1-Carboxy-3-butylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (2',3'-Ketal of Ribavirin with Sodium Levulinate, 5). A mixture of 521 mg (1.41 mmol) of ketal ester 37 dissolved in 5.4 ml of methoxyethanol and 4.5 ml of 1 M potassium carbonate solution (4.5 mmol) was heated under argon in a 75°C oil bath for 2 h. The solvent was removed by evaporative distillation (0.025 mm Hg, < 60°C) using a Kügelrohr apparatus to give a solid, which was dissolved in 15 ml of water. The aqueous solution was chromatographed on a DEAE Sephadex A-25 (HCO_3^- form) column (1.5 × 23 cm) with elution with 200 ml of water, followed by a 0 to 0.5 M ammonium bicarbonate gradient (800-ml volume), and collection of 10-ml fractions at a flow rate of 2.5 ml/min. Monitoring by UV indicated that Fractions 7 to 16 contained the major peak. These pooled fractions were lyophilized, dissolved in 100 ml of water, and lyophilized again. The very hygroscopic ammonium salt that was obtained was dissolved in 10 ml of water. This solution was chromatographed three times on a Dowex 50W-X4 (Na^+ form) column (2.5 × 18 cm) using water as the eluant and collecting 20-ml fractions. The first Dowex column gave a broad peak. The product was in Fractions 3 to 10. The second and third Dowex columns gave much sharper peaks, with the product being located in Fractions 2 to 4. Lyophilization afforded 325 mg (63% yield) of ketal sodium salt 5 as a fluffy white solid, mp 135-137°C; HPLC t_R 5.24 min (92%, major diastereomer), 9.60 min (8%, minor diastereomer); $[\alpha]_D^{23}$ -32.5° (7.75 × 10^{-3}, water); IR (Nujol mull) 2400-3600, 1700 (shoulder), 1680, 1560, 1300, 1080, 890, 720 cm^{-1}; 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 1.24 (s, 2.7 H, CH_3C) (major

diastereomer), 1.41 (s, 0.3 H, CH_3C) (minor diastereomer), 2.80-3.10 (m, 4 H, CH_2CH_2), 3.44 (m, 2 H, 5'-CH_2), 4.23 (t, J = 6 Hz, 1 H, 4'-CH), 4.88 (d, J = 6 Hz, 1 H, 3'-CH), 5.16 (d, J = 6 Hz, 1 H, 2'-CH), 6.19 (s, 1 H, 1'-CH), 7.65 (broad, 1 H, NH), 7.86 (broad s, 1 H, NH), 8.82 (s, 1 H, 5-HetH); ^{13}C NMR (dimethyl sulfoxide-d_6) 33.4 (major diastereomer), 33.7 (minor diastereomer), 42.5 (major diastereomer), 43.3 (minor diastereomer), 46.2, 71.3, 91.5 (major diastereomer), 91.8 (minor diastereomer), 94.0, 98.3, 102.9, 124.5 (major diastereomer), 124.9 (minor diastereomer), 155.3, 167.3, 170.4, 186.8 ppm. Anal. calcd for $C_{13}H_{17}N_4NaO_7$: C 42.86, H 4.70, N 15.38; found: C 42.53, H 5.09, N 15.58.

(7422-80)

1-[2,3-O-(1-Carbethoxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (2',3'-Ketal of Ribavirin with Ethyl 4-Acetylbutyrate, 38). To a stirred solution of 8.41 g (53 mmol) of ethyl 4-acetylbutyrate (36) and 17.8 g (120 mmol) of triethyl orthoformate (13) was added under argon 2.00 g (8.19 mmol) of ribavirin (1). This mixture was cooled to 0°C while 0.66 ml of 70% perchloric acid was added dropwise with rapid stirring. The reaction mixture was stirred at ambient temperature for 4 h, at which time TLC (10% methanol/chloroform) indicated almost complete conversion of ribavirin (R_f 0.07) to ketal 38 (R_f 0.50) and unreacted ethyl 4-acetylbutyrate (R_f 0.99). The reaction mixture was dissolved in 50 ml of chloroform and washed with 50 ml of 5% sodium bicarbonate solution containing 5 ml of ethanol to break up the emulsion. The chloroform layer was dried

(sodium sulfate) and concentrated at reduced pressure to an oil, which was treated at 4°C for 18 h with 100 ml of hexanes. The residue after trituration was purified by flash column chromatography (0-10% methanol/chloroform gradient with incremental 2% methanol steps of 300, 500, 300, 600, and 300 ml, and 25-ml fractions collected beginning at 6% methanol addition) to give 1.76 g (56% yield) of ketal <u>38</u> as a white foam. The foam was crystallized from dichloromethane/ether and dried to give 777 mg (25% yield) of ketal <u>38</u> as white crystals, mp 100-104°C; $[\alpha]_D^{23}$ -40.3° (7.5 × 10^{-3}, ethanol); IR (Nujol mull) 3370, 3125, 1730, 1700, 1665, 1640, 1305, 1235, 1189, 1120, 1090, 1070, 1030, 1010, 850, 765, 740, 720 cm^{-1}; 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 1.26 (s, 2.4 H, CH_3C) (major diastereomer), 1.43 (s, 0.6 H, CH_3C) (minor diastereomer), 1.80-2.00 and 2.50-2.80 (2 m, 6 H, $(CH_2)_2CH_2CO_2$), 3.45 (m, 2 H, 5'-CH_2). 4.24 (m, 1 H, 4'-CH), 4.88 and 5.18 (2 dd, J = 6 Hz, J = 2 Hz, 2 H, 2',3'-CH), 6.20 (d, J = 2 Hz, 1 H, 1'-CH), 7.64 (broad s, 1 H, NH), 7.86 (broad s, 1 H, NH), 8.80 (s, 0.2 H, 5-HetH) (minor diasteromer), 8.82 (s, 0.8 H, 5-HetH) (major diasteromer); ^{13}C NMR (dimethyl sulfoxide-d_6) 24.1, 28.7 (major diastereomer), 29.7 (minor diastereomer), 33.2 (major diastereomer), 34.8 (minor diastereomer), 43.3, 47.6, 69.7, 71.3, 91.6 (major diastereomer), 91.8 (minor diastereomer), 94.1 (major diastereomer), 94.3 (minor diastereomer), 98.5, 102.6, 123.8 (major diastereomer), 124.2 (minor diastereomer), 155.2, 167.4, 170.2, 182.8 ppm. Anal. calcd for $C_{16}H_{24}N_4O_7$: C 50.00, H 6.29, N 14[illegible]8; found: C 49.93, H 6.32, N 14.44.

(7422-4,56,78)

1-[2,3-O-(1-Carboxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (2',3'-Ketal of Ribavirin with Sodium 4-Acetylbutyrate, 7). A mixture of 614 mg (1.60 mmol) of ketal ester 38 dissolved in 6.2 ml of methoxyethanol and 5.2 ml of 1 M aqueous potassium carbonate (5.2 mmol) was heated under argon in a 75°C oil bath for 2 h. The solvent was then removed by evaporative distillation (0.025 mm Hg, < 60°C) using a Kűgelrohr apparatus. The solid residue was dissolved in 15 ml of water and chromatographed on a DEAE Sephadex A-25 (HCO_3^- form) column (1.5 × 23 cm) using 200 ml of water as the eluant, followed by a linear gradient of 0 to 0.5 M ammonium bicarbonate (800-ml volume) at a flow rate of 2.5 ml/min. Fractions (20-ml volume) were monitored by UV. Material eluting in Fractions 9 to 17 (major peak) was lyophilized, dissolved in 100 ml of water, and relyophilized to give the hygroscopic ketal ammonium salt, which was dissolved in 10 ml of water and passed three times through a Dowex 50W-X4 (Na^+ form) column (2.5 × 18 cm) using water as the eluant. Fractions 3 to 7 contained the major peak in all three columns and were lyophilized after the third column to give 567 mg (93% yield) of the ketal sodium salt 7 as a fluffy white solid, mp 138-140°C; $[\alpha]_D^{23}$ -31.9° (9.1 × 10^{-3}, water); IR (Nujol mull) 2500-3600, 1700 (shoulder), 1680, 1550, 1290, 1180, 1060, 900, 860, 707 cm^{-1}; 90 MHz ^1H NMR (dimethyl sulfoxide-d_6) δ 1.26 (s, 2.4 H, CH_3C) (major diastereomer), 1.43 (s, 0.6 H, CH_3C) (minor diastereomer), 2.50-3.00 (m, 6 H, $(CH_2)_3$), 3.45 (m, 2 H, 5'-CH_2), 4.23 (m, 1 H, 4'-CH), 4.89 and 5.18 (2 dd, J = 6 Hz, J = 2Hz, 2 H, 2',3'-CH), 6.20 (d,

J = 2 Hz, 1 H, 1'-CH), 7.64 (broad s, 1 H, NH), 7.86 (broad s, 1 H, NH), 8.80 (s, 0.2 H, 5-HetH) (minor diastereomer), 8.82 (s, 0.8 H, 5-HetH) (major diastereomer); ^{13}C NMR (dimethyl sulfoxide-d_6) 30.7 (major diastereomer), 31.4 (minor diastereomer), 33.4 (major diastereomer), 34.2 (minor diastereomer), 48.3, 48.6, 71.4, 91.5 (major diastereomer), 92.0 (minor diastereomer), 94.0 (major diastereomer), 94.4 (minor diastereomer), 98.3 (major diastereomer), 98.5 (minor diastereomer), 102.8, 124.5 (major diastereomer), 124.9 (minor diastereomer), 155.4, 167.4, 170.4, 187.2 ppm. Anal. calcd for $C_{14}H_{19}N_4NaO_7 \cdot 0.5H_2O$: C 43.41, H 5.20, N 14.46; found: C 43.41, H 5.38, N 14.34.

(7422-58,62,68,82)

[redacted] modified. [redacted] disulfide (40) in 68 ml of anhydrous ethanol, which had been prepared by heating the mixture, was added at room temperature 5.35 g (58.0 mmol) of 3-mercaptopropanol (39) (Phillips 66 Company, Bartlesville, Oklahoma 74004), and this mixture was stirred at room temperature for 21 h under argon. The reaction mixture was concentrated at reduced pressure to give a viscous yellow oil from which pyridyl-2-mercaptan (42) crystallized on standing. The solid (6.2 g) was removed by trituration with six 50-ml portions of 3:2 ether/hexanes. The solvent-soluble material was concentrated at reduced pressure to give a slightly yellow oil, which was purified by flash column chromatography (25-67% ethyl acetate/hexanes in 300-ml gradient steps of 8% ethyl

acetate, 125-ml fractions) to remove trace impurities, excess 2,2'-dipyridyl disulfide, and pyridyl-2-mercaptan; TLC (80% ethyl acetate/hexanes, potassium permanganate spray) R_f 0.27 (pyridyl-2-mercaptan), 0.51 (disulfide 41), 0.64 (3-mercaptopropanol), and 0.72 (2,2'-dipyridyl disulfide). Flash column chromatography (25-67% ethyl acetate/hexane, 8% ethyl acetate increments of 300 ml, with 125-ml fractions being collected) afforded, after concentration of the fractions at reduced pressure, 6.93 g (59% yield) of disulfide 41 as a clear, colorless oil; IR (film) 3340, 3045, 2930, 2880, 1585, 1565, 1450, 1420, 1280, 1265, 1150, 1125, 1050, 1000, 910, 765, 725 cm^{-1}; 90 MHz ^{1}H NMR (deuteriochloroform) δ 1.95 (m, 2 H, $CH_2\underline{CH_2}CH_2$), 2.97 (t, J = 7 Hz, 2 H, CH_2S), 3.76 (t, J = 6 Hz, 2 H, CH_2O), 3.98 (s, 1 H, OH), 7.0-7.15 (m, 1 H, 3-HetH), 7.50-7.65 (m, 2 H, 4,5-HetH), 8.41 (broad d, J = 5 Hz, 1 H, 6-HetH).

(7421-40; 7422-96)

1-(5-O-Phosphato-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide Diammonium Salt (Ribavirin 5'-Phosphate Diammonium Salt, 43). A literature procedure[10] for the preparation of the 5'-phosphate of ribavirin was modified.

Procedure A. To 7.5 ml of trimethyl phosphate (Aldrich, distilled) containing 617 mg (4.08 mmol) of phosphorous oxychloride was added 305 mg (1.25 mmol) of ribavirin (1). The reaction mixture was rapidly stirred at 4°C for 12 h at which time the ribavirin had dissolved. The reaction mixture was then poured into 15 ml of ice

acetate, 125-ml fractions) to remove trace impurities, excess 2,2'-dipyridyl disulfide, and pyridyl-2-mercaptan; TLC (80% ethyl acetate/hexanes, potassium permanganate spray) R_f 0.27 (pyridyl-2-mercaptan), 0.51 (disulfide 41), 0.64 (3-mercaptopropanol), and 0.72 (2,2'-dipyridyl disulfide). Flash column chromatography (25-67% ethyl acetate/hexane, 8% ethyl acetate increments of 300 ml, with 125-ml fractions being collected) afforded, after concentration of the fractions at reduced pressure, 6.93 g (59% yield) of disulfide 41 as a clear, colorless oil; IR (film) 3340, 3045, 2930, 2880, 1585, 1565, 1450, 1420, 1280, 1265, 1150, 1125, 1050, 1000, 910, 765, 725 cm^{-1}; 90 MHz ^1H NMR (deuteriochloroform) δ 1.95 (m, 2 H, $CH_2\underline{CH_2}CH_2$), 2.97 (t, J = 7 Hz, 2 H, CH_2S), 3.76 (t, J = 6 Hz, 2 H, CH_2O), 3.98 (s, 1 H, OH), 7.0-7.15 (m, 1 H, 3-HetH), 7.50-7.65 (m, 2 H, 4,5-HetH), 8.41 (broad d, J = 5 Hz, 1 H, 6-HetH).

(7421-40; 7422-96)

<u>1-(5-O-Phosphato-β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide Diammonium Salt (Ribavirin 5'-Phosphate Diammonium Salt, 43)</u>. A literature procedure[10] for the preparation of the 5'-phosphate of ribavirin was modified.

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water and its pH was adjusted to 2 with 2 M sodium hydroxide. This mixture was washed with two 25-ml portions of chloroform to remove trimethyl phosphate. The aqueous layer was gently stirred with 7.5 g of activated charcoal, which had been washed with water, until no carbohydrate-containing material was present in the aqueous supernatant (anisaldehyde spray detection). The charcoal was transferred to a sintered-glass funnel and washed with water until the rinse liquid tested free of ions, as determined by conductance (8.5×10^{-5} Ω^{-1}). The charcoal was then washed with 10:10:1 ethanol/water/concentrated ammonium hydroxide (approximately 100 ml) to remove the product. The filtrates (detected by anisaldehyde spray on TLC plates) were pooled and lyophilized. The solid was dissolved in 5 ml of 5% ammonium hydroxide. Because attempted precipitation of the product by ethanol failed, the lyophilized material was purified by chromatography on a DEAE Sephadex A-25 (HCO_3^- form) column (1.5 × 23 cm) using a linear gradient of 0.0 to 0.3 M ammonium bicarbonate solution (1.4-L volume) at a flow rate of 2.3 ml/min with collection of 18-ml fractions. Fractions 32 to 37 (major peak) were lyophilized, dissolved in 100 ml of water, and lyophilized again to give 32 mg (4%) of ribavirin 5'-phosphate diammonium salt (43) as a white solid, mp 194-200°C (dec); 90 MHz ^{1}H NMR (dimethyl sulfoxide-d_6) δ 3.80 (m, 2 H, 5'-CH_2), 3.95-4.30 (m, 2 H, CH), 4.45 (t, J = 4 Hz, 1 H, CH), 4.91 (broad s, 10 H, NH_4^+ and OH), 5.78 (d, J = 4 Hz, 1 H, 1'-CH), 7.63 (broad s, 1 H, NH), 7.94 (broad s, 1 H, NH), 8.92 (s, 1 H, 5-HetH).

(7421-58)

<u>Procedure B</u>. A 1.22-g (5.00-mmol) portion of ribavirin (<u>1</u>) was dissolved in 50 ml of hot pyridine, and the resultant solution was concentrated to dryness. The dried white solid was pulverized and added to a cold (0°C), stirred solution of 1.5 ml (16.1 mmol) of phosphorous oxychloride (Aldrich Gold Label) in 30 ml of triethyl phosphate, which had been dried over 3Å molecular seives. The reaction mixture was stirred at 0°C with monitoring by TLC (70% acetonitrile/0.1 M ammonium chloride). A drop of the reaction mixture was diluted with an equal volume of water, and the resultant mixture spotted on a silica gel plate. After the plate was developed in the aforementioned solvent system, the spots were detected by spraying with vanillin solution, which had been prepared according to the anisaldehyde recipe given under General Procedures and Instrumentation, and with 5% phosphomolybdic acid in ethanol, followed by heating. After a reaction time of 5 h, the spot (R_f 0.62) corresponding to <u>1</u> was no longer evident, but spots corresponding to <u>43</u> (R_f 0.31) and triethyl phosphate (R_f 0.83) were present. At 5.5 h, the reaction mixture was poured onto 60 g of ice, and this mixture was diluted with 3 M sodium hydroxide until the pH reached 7.0. Triethyl phosphate was removed by two washings with 100-ml portions of chloroform. The aqueous layer was then put under reduced pressure to remove residual traces of chloroform and lyophilized to give 6.3 g of a white solid. The 90 MHz ^1H NMR spectrum (deuterium oxide) of this solid showed nonexchangeable proton multiplets at 4.16-4.72 ppm, a sharp doublet at 6.18 ppm (1'-CH), and a sharp singlet at 8.93 ppm (5-HetH).

The solid was dissolved in 50 ml of water, and the pH was readjusted from 1.3 to 2.0 with 3 M sodium hydroxide. This solution was stirred with several incremental portions of activated Barnaby-Cheney UU 1064 carbon until TLC indicated that 43 was no longer present in the solution. The 30 g of activated carbon had been prepared by successive washings with 0.1 M hydrochloric acid, 0.1 M ammonium hydroxide, 60% aqueous ethanol, water, 0.1 M hydrochloric acid, and water (100-ml volumes). After adsorption of the nucleotide, the carbon was suction-filtered and washed with five 50-ml portions of water until constant conductivity was attained. Each aqueous filtrate was monitored by TLC and conductivity measurement. The desalted nucleotide was recovered from the carbon by stirring with five 50-ml portions of 10:10:1 ethanol/water/concentrated ammonium hydroxide, followed by suction filtration. Monitoring the filtrates by TLC indicated that 43 was present in the first three fractions, which were combined and concentrated at reduced pressure to remove the ethanol. The residual solution was lyophilized to give 0.9 g of an amorphous solid, which was solubilized in approximately 3 ml of 6 M ammonium hydroxide; ethanol was then added until no further turbidity occurred. A white gum separated out. The gum produced 841 mg of filterable solid upon trituration at ice-bath temperatures with acetonitrile (about 2 ml) and intermittent warming. TLC examination of the solid indicated a contaminant at the origin. Therefore, the solid was recombined with its mother liquors, evaporated, and lyophilized.

The residue was dissolved in the minimum amount of water, and the solution was filtered through Whatman GFC paper to remove traces of carbon, brought to pH 8 with ammonium hydroxide, and chromatographed on 6 g of DEAE Sephadex A-25 (HCO_3^- form) column (0.9 × 47.5 cm), eluting first with 180 ml of water at a flow rate of 1 ml/min, followed by a linear gradient of 0-0.3 M ammonium bicarbonate (2000 ml) at a flow rate of 1.25 ml/min, with 15-ml fractions being collected and monitoring at 254 nm. All peaks were collected and lyophilized to constant weight (three to four times from water). Fractions 3 to 5 of the water eluate contained 25 mg of ribavirin. Fractions 27 to 32 of the gradient eluate contained 210 mg of 43, which was homogenous by TLC (7:1:2 isopropyl alcohol/ammonium hydroxide/water, vanillin spray detection) R_f 0.10. Fractions 33 to 41 afforded 300 mg of material, which was predominately 43 with a minor contaminant as indicated by TLC R_f 0.45. In contrast, 1 had R_f 0.63 in this solvent system. Fractions 42 to 44 appeared as a small side peak and on lyophilization gave 12 mg of material, which by TLC contained an appreciable amount of R_f 0.45 product as well as 43.

The 300-mg fraction was dissolved in 6 M ammonium hydroxide. The solution was treated with ethanol to precipitate the product. After 18 h at 4°C followed by warming to room temperature, 296 mg of a white solid was collected in two portions. The solid was homogenous by TLC and was combined with the 210 mg of material isolated from the column, dissolved in 6 M ammonium hydroxide, and lyophilized to give 494 mg

(28% yield) of 43 as a white solid. This material cospotted on TLC with that prepared by Procedure A.

(7747-1)

[1-^{3}H]1,2,3,5-Tetra-O-acetyl-β-D-ribofuranose (51). A literature procedure[4] was modified. [1-^{3}H]D-Ribose (8.5 mCi, specific activity 15 Ci/mmol; ARC) in 9:1 ethanol/water was concentrated from three 4-ml portions of methanol to leave a film on the inside of a 100-ml round-bottomed flask. To this residue was added 1.00 g (6.67 mmol) of unlabeled D-ribose. This mixture was dissolved with stirring in 16 ml of methanol under argon. The clear solution was cooled to 0°C before 80 μl of concentrated sulfuric acid was added dropwise. After being stirred at 4°C for 12 h, the reaction mixture was neutralized with 3 ml of pyridine and concentrated at reduced pressure (15 mm Hg) to give the α,β-methyl-riboside mixture (50) as a thick, brown oil. The oil was dissolved in 3.0 ml of acetic acid, and then 4.0 ml (36 mmol) of acetic anhydride was added. After cooling to 0°C under argon, the solution was treated with 120 μl of concentrated sulfuric acid and then was allowed to come to ambient temperature over a period of 1 h. The reaction mixture was then cooled to 0°C, treated with another 200-μl portion of concentrated sulfuric acid, and then allowed to return to room temperature over a period of 2 h. Next, 1.20 g (14.6 mmol) of anhydrous sodium acetate in 30 ml of ethanol was added. This mixture was concentrated to dryness below 50°C. The residue was reconcentrated from three 35-ml portions of ethanol and then dissolved in 25 ml of chloroform. The chloroform solution was washed with 20 ml

of water, dried (magnesium sulfate), and concentrated to a viscous brown oil, which was dried (0.03 mm Hg). The product was dissolved in 5 ml of hot ethanol, but on cooling to -20°C did not crystallize when seeded with an authentic sample of cold 15. The material was concentrated, and the residue was dissolved in 3 ml of chloroform and chromatogrephed on silica gel (100-ml wet volume in chloroform) using a step gradient of 1 to 5% methanol/chloroform (1% methanol increments of 50-ml volume) and collecting 15-ml fractions. Fractions 8 and 9 gave after concentration 0.19 g (9% yield) of [1-^{3}H]methyl-2,3,5-tri-O-acetyl-β-D-ribofuranoside, which showed one spot (R_f 0.64) on TLC (5% methanol/chloroform). Fraction 10 gave after concentration a mixture of 0.24 g of this material and a more polar byproduct (R_f 0.41). Frections 11 and 12 contained 0.18 g of the more polar material. This polar material was tentatively identified as [1-^{3}H]methyl-2,3,5-tri-O-acetyl-α-D-ribofuranoside. Incomplete removal of pyridine before the acetylation step could have afforded these compounds.[4] Therefore, the acetylation procedure was repeated.

A solution of 0.19 g (0.655 mmol, 0.603 mCi) of [1-^{3}H]methyl-2,3,5-tri-O-acetyl-β-D-ribofuranoside, 1.0 ml of acetic anhydride, and 0.75 ml of glacial acetic acid was cooled in an ice bath and treated with 30 μl of concentrated sulfuric acid. The resulting solution was stirred at ambient temperature for 1 h and then was recooled in an ice bath while 50 μl of concentrated sulfuric acid was added. The resulting solution was stirred at room temperature for 2 h, and then treated with 0.25 g of anhydrous sodium acetate. The mixture was

coevaporated with three 5-ml portions of ethanol. The residue was dissolved in 10 ml of chloroform, washed with two 5-ml portions of water and 5 ml of brine, and dried over anhydrous potassium carbonate. Evaporation at reduced pressure afforded 0.23 g of a colorless oil.

A solution of 0.42 g (1.45 mmol, 1.56 mCi) of [1-^{3}H]methyl-2,3,5-tri-_O_-acetyl-α-_D_-ribofuranoside, 2 ml of acetic anhydride, and 1.5 ml of glacial acetic acid was cooled with an ice bath while being treated with 60 μl of concentrated sulfuric acid. The resulting solution was stirred at room temperature for 1 h and then recooled in an ice bath while 100 μl of concentrated sulfuric acid was added. The resulting solution was stirred at ambient temperature for 2 h and was then treated with 0.5 g of anhydrous sodium acetate. This mixture was coevaporated with three 5-ml portions of ethanol. The residue was dissolved in 10 ml of chloroform, washed with two 5-ml portions of water and 5 ml of brine, and dried over anhydrous potassium carbonate. Evaporation left 0.56 g of an oil. This oil was combined with the 0.23 g of oil obtained above for purification by crystallization. The ratio of β:α anomers in this mixture was approximately 4:1 as determined by the TLC (3:1 ether/cyclohexane) spot diameters of _51_ (R_f 0.45) and its α-anomer (R_f 0.38).

(7421-61)

The 0.79 g of crude tritiated tetra-acetate mixture was dissolved in 1 ml of ethanol and seeded with 15. Crystallization occurred at -20°C to give 0.50 g (22% yield) of 51 as white crystals. The mother liquor failed to give additional material, most likely because of the large amount (50%) of α-anomer present. The mother liquor was filtered through 1 g of silica gel using 7 ml of 75% ether/cyclohexane. Evaporation afforded 315 mg of 1:1 mixture of 51 and its α-anomer.

(7421-62)

[1-^{3}H]3-Cyano-1-(2,3,5-tri-*O*-acetyl-β-*D*-ribofuranosyl)-1,2,4-triazole (52). A mixture of 0.50 g (1.57 mmol, 1.04 mCi) of [1-^{3}H]-1,2,3,5-tetra-*O*-acetyl-β-*D*-ribofuranosyl (51) and 148 mg (1.57 mmol) of 3-cyano-1,2,4-triazole (14) was heated at 150-160°C under argon. After the mixture melted, approximately 2 mg of bis(4-nitrophenyl) phosphate (16) was added. The resulting solution was stirred under reduced pressure at 160°C for 15 min. Next, the material was allowed to cool at ambient temperature and was then stirred vigorously with 5 ml of chloroform. The resulting mixture was filtered through Norit A and evaporated leaving 0.62 g of an oil. The oil was dissolved with heating in 2 ml of ether. After seeding with 17, crystallization was allowed to occur overnight giving 247 mg (52% yield) of 52. The mother liquors were reduced to half their volume and seeded, but failed to produce further crystalline material.

(7421-63)

[1-^{3}H]3-Cyano-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1,2,4-triazole (52) from 51 and Its α-Anomer. A 1:1 mixture of 0.32 g (1.00 mmol) of 51 and its α-anomer and 93 mg (0.99 mmol) of 3-cyano-1,2,4-triazole (14) were heated at 150-160°C under argon. After the mixture melted, approximately 2 mg of bis(4-nitrophenyl) phosphate (16) was added, and the resulting solution was stirred at 150-160°C under vacuum (40 mm Hg) for 15 min. After cooling to ambient temperature, the residue was vigorously stirred with 2 ml of chloroform. The resulting mixture was filtered through Norit A. The filtrate was concentrated at reduced pressure to give 0.49 g of an oil, which was dissolved with heating in 1.5 ml of ether. The solution was seeded with 17 to induce crystallization at room temperature for 3 h, giving 98.5 mg (32% yield) of pure 52 as white crystals. The mother liquors were concentrated at reduced pressure to one-half of their volume and seeded with 17, but failed to give additional crystalline material.

(7421-64)

[1-^{3}H]1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide ([1'-^{3}H]-Ribavirin, 9). A solution of 392 mg (1.11 mmol, 1.03 mCi) of [1-^{3}H]-3-cyano-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1,2,4-triazole (52) in 3.0 ml of concentrated ammonium hydroxide was heated in an oil bath to 100-105°C for 1 h. Next, the reaction mixture was evaporated to dryness at reduced pressure on a rotary evaporator, diluted with 6 ml of anhydrous ethanol, and filtered through decolorizing carbon. The volume of the filtrate was reduced to one-half by concentration at reduced pressure, leading to precipitation of the product as a white

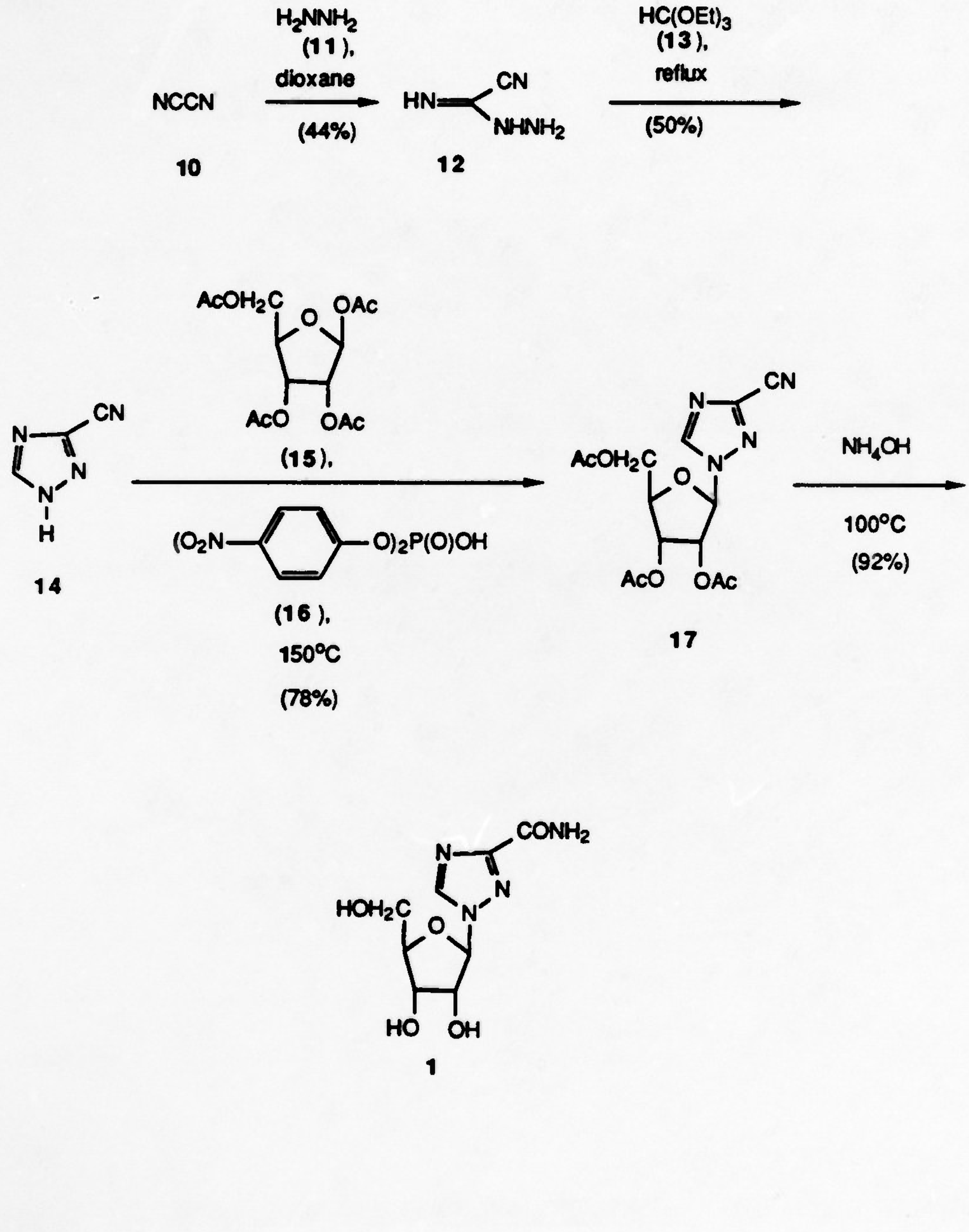
solid. This mixture was heated to solution and, after cooling to room temperature, was seeded with cold ribavirin (1). Crystallization was induced by cooling at 4°C. The mother liquor was concentrated to one-half of its volume and seeded. This process was repeated several times. The crops of crystals were combined to give 140 mg (52% yield) of 9 which was 97.5% radiopure (0.457 mCi, specific activity 0.82 mCi/mmol) by HPLC with t_R 9.0 min, using a 10-μ RadialPak 8C18 column cartridge with water as the eluate at a flow rate of 1.0 ml/min and UV detection at 220 nm and radiodetection by 2AUFS ratemeter at 1×10^3 (20% split) with Ecolite cocktail and 0.2-inch/min chart speed. This sample of 9 was stored frozen in 5 ml of water.

(7421-66)

C. Results

Until a supply of ribavirin (1) was received from Dr. Meir Kende (Department of Antiviral Studies, USAMRIID), to facilitate synthetic work we prepared 1 using the literature procedure[1] outlined in Scheme I-1. Cyanogen (10) was allowed to react with hydrazine (11) in dioxane to give 1-cyanoformimidic acid hydrazide (12) in 44% yield on recrystallization from isopropyl alcohol/hexane. Hydrazide 12 was heated at reflux temperature with excess triethyl orthoformate (13) to

Scheme I-1. Synthesis of Ribavirin

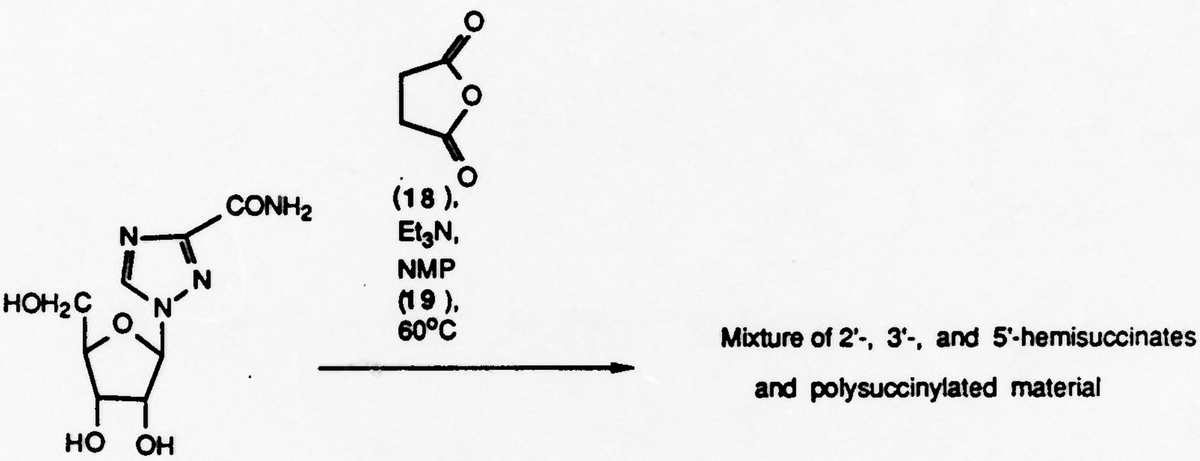


give 3-cyano-1,2,4-triazole (14) in 78% yield after crystallization from ethyl acetate/benzene. A mixture of triazole 14 and 1,2,3,5-tetra-O-acetyl-β-D-ribofuranose (15) was heated at 150°C (external temperature) in the presence of bis(4-nitrophenyl) phosphate (16) as the catalyst, with concomitant removal of the acetic acid byproduct at reduced pressure to afford a 71% yield of the triacetate 17 after recrystallization from ether. Hydrolysis of the three acetate groups and the nitrile group of 17 with ammonium hydroxide at 100°C followed by recrystallization from ethanol gave a 78% yield of ribavirin. The physical data—melting point, and infrared (IR) and proton nuclear magnetic resonance (^1H NMR) spectra—obtained for 12, 14, 17, and 1 were in agreement with those reported in the literature.[1,2]

The next synthetic target was the 5'-hemisuccinate ester (2) of ribavirin. The carboxyl group of the hemisuccinate would be used to link 2 to MAbs by formation of an amide bond with the ε-amino groups of the lysines of the MAbs. Several routes for the formation of 2 were explored before success was achieved. Direct reaction of succinic anhydride (18) with ribavirin in dimethylformamide has been reported to give 2.[8] Therefore, ribavirin was allowed to react with 1.0 equivalent of 18 in N-methylpyrrolidinone (19) at 60°C for 1 hour (Scheme I-2). The resulting reaction mixture was purified on DEAE Sephadex A-25 (0.0-0.5 M ammonium bicarbonate gradient) to give three fractions: 1) unreacted 1, 2) succinylated 1, and 3) polysuccinylated material. The second fraction proved to be a mixture of the 2'-, 3'-, and 5'-hemisuccinates according to the ^1H NMR spectrum, which showed

three signals at 8.82*, 8.87*, and 8.90 ppm for the 5-proton on the triazole ring in a ratio of 1.0*:1.3*:3.6, respectively. The nonselectivity of the succinylation may have been caused by the temperature at which it was conducted. Unfortunately, lower reaction temperatures did not dissolve 1 and therefore no reaction occurred.

Scheme I-2. Attempted Synthesis of Ribavirin 5'-Hemisuccinate



1

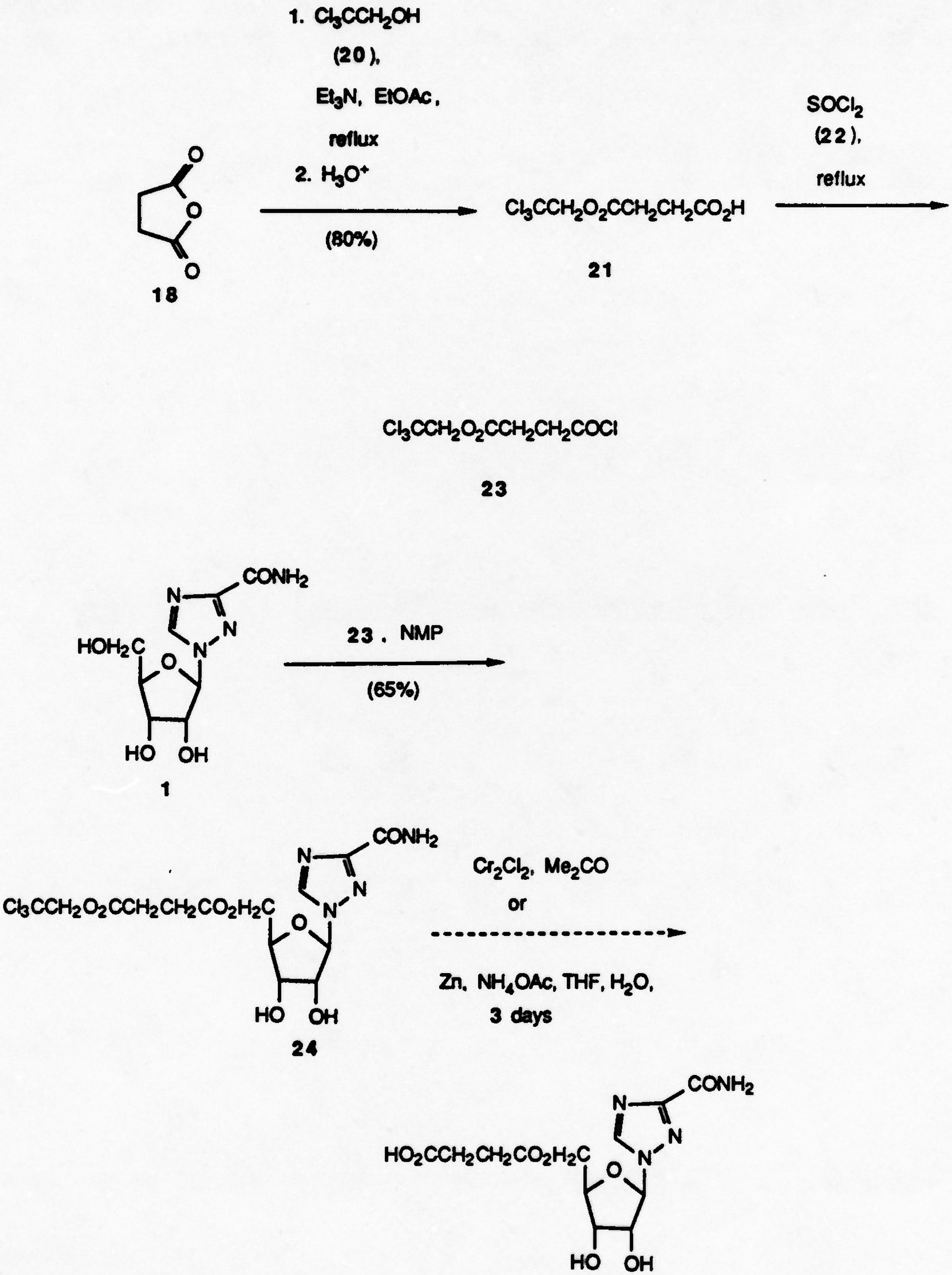
The second approach to 2 involved the selective acylation of the primary 5'-hydroxyl group of 1 using an activated succinate ester, which had one carboxyl group protected by a readily cleavable ester group. This approach was based on the report[11] that acid chlorides of succinate half esters react specifically with the 5'-hydroxyl group of

*Assignments may be reversed.

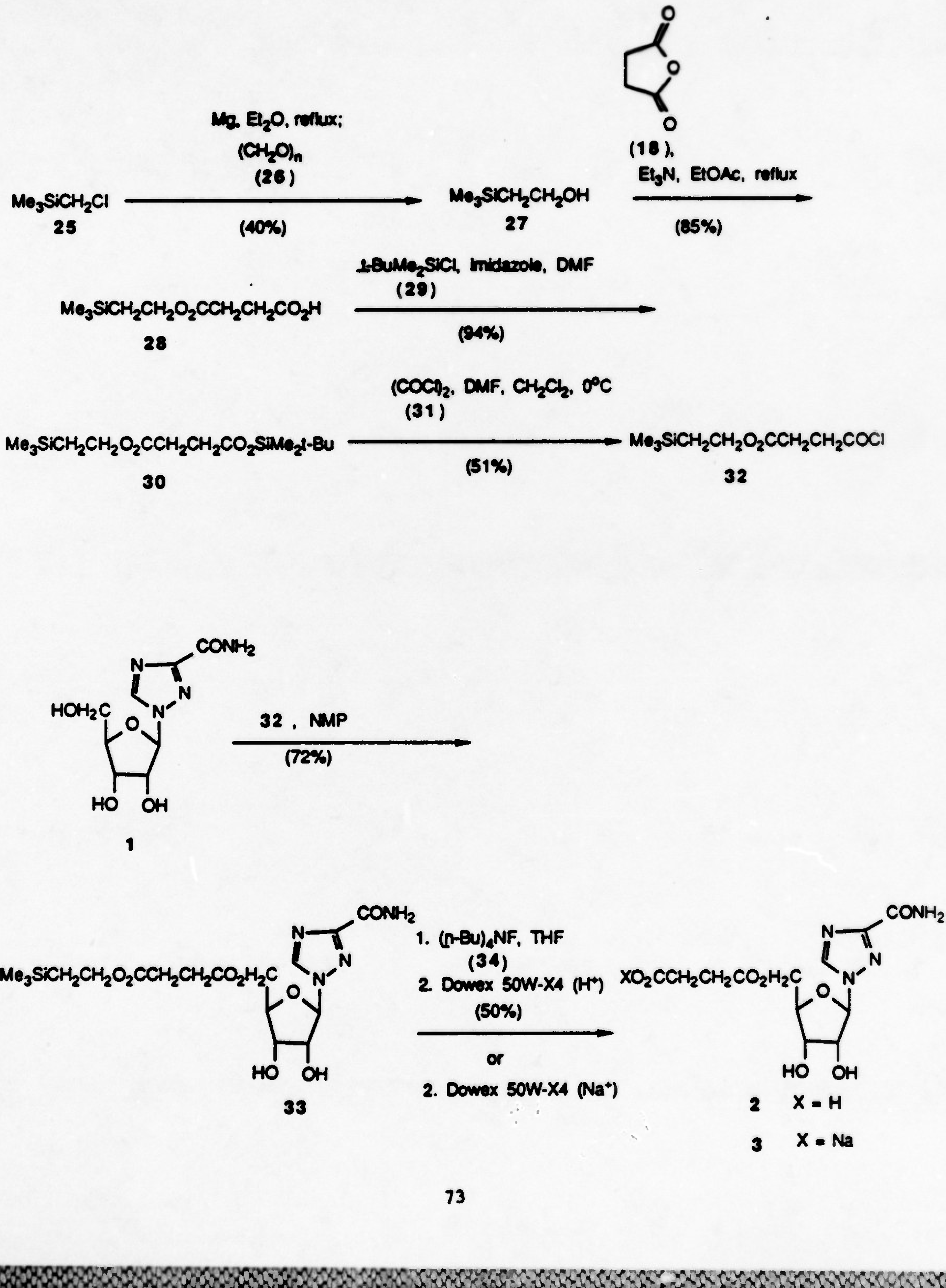
other nucleosides. The 2,2,2-trichloroethyl ester protecting group was investigated first because this group can be readily cleaved under mild reductive conditions. Reaction of succinic anhydride (18) with 2,2,2-trichloroethanol (20) in refluxing ethyl acetate in the presence of 1.0 equivalent of triethylamine as the base gave the hemisuccinate 21 in 80% yield after recrystallization from chloroform/hexane (Scheme I-3). The hemisuccinate 21 was then converted to the ester acid chloride 23 by treatment with 9.0 equivalents of thionyl chloride (22) at reflux for 1 hour. Acid chloride 23 was not purified but was immediately allowed to react with 1 in N-methylpyrrolidinone. The primary ester (24, mp 109.5-111°C) that formed was isolated in 65% yield after flash column chromatography on silica gel using 0-9% methanol/chloroform. The structure of 24 was confirmed by its IR and ^{1}H NMR spectra and elemental analysis. The next step in the synthesis was cleavage of the protecting group. Reductive cleavage with chromous chloride in acetone[12] gave a complex mixture of products, whereas reductive cleavage with zinc in aqueous ammonium acetate/-tetrahydrofuran[13] for three days gave a small amount of the desired product (2) and another compound from which it could not be separated by ion-exchange chromatography on DEAE Sephadex A-25 (0.0-0.5 M ammonium bicarbonate gradient).

The next protecting group investigated was the 2-(trimethyl-silyl)ethyl ester group, which is another readily cleavable group. Use of this group resulted in a successful synthesis of 2 (Scheme I-4). This route was similar to that employed for the preparation of

Scheme I-3. Second Attempted Synthesis of Ribavirin 5'-Hemisuccinate



Scheme I-4. Syntheses of Ribavirin 5'-Hemisuccinate and Its Sodium Salt



succinate 24. Trimethylsilylmethyl chloride (25) was converted to the Grignard reagent by treatment with magnesium in refluxing ether. Reaction of the Grignard reagent with paraformaldehyde (26) followed by an aqueous ammonium chloride workup afforded 2-(trimethylsilyl)-ethanol (27) in 40% yield. Heating 27 for two hours with succinic anhydride (18) in refluxing ethyl acetate containing 1.0 equivalent of triethylamine as the base gave an 85% yield of 2-(trimethylsilyl)ethyl hemisuccinate ester (28) as a colorless oil.

In contrast to the 2,2,2-trichloroethyl ester protecting group, the 2-(trimethylsilyl)ethyl protecting group was too labile to survive the direct conversion of the hemisuccinate to the acid chloride. Both thionyl chloride and oxalyl chloride, even in the presence of triethylamine as a base, cleaved the 2-(trimethylsilyl)ethyl protecting group. For example, treatment of 28 with thionyl chloride gave succinic anhydride, presumably from protodesilylation by the hydrogen chloride produced from formation of the acid chloride. Because the direct conversion was not possible, a stepwise procedure was employed. The t-butyldimethylsilyl ester (30) of 28 was prepared in 94% yield using t-butyldimethylsilyl chloride (29) and imidazole in dimethylformamide at room temperature for 24 hours.[14] The t-butyldimethylsilyl ester was readily converted to the acid chloride 32 in 51% yield under aprotic conditions, using oxalyl chloride (31) in methylene chloride at 0°C for 1.5 hours.[7]

Treatment of 1 with acid chloride 32 in N-methylpyrrolidinone for 16 hours at room temperature and purification by chromatography on silica gel with 0-10% methanol/chloroform gave the 5'-succinate ester 33 in 72% yield. Removal of the 2-(trimethylsilyl)ethyl protecting group with tetra(n-butyl)ammonium fluoride (34) in tetrahydrofuran at room temperature for 18 hours, followed by DEAE Sephadex A-25 (0.0-05 M ammonium bicarbonate gradient) and Dowex 50W-X4 resin (H^+) ion-exchange chromatographies gave ribavirin 5'-hemisuccinate (2) in 50% isolated yield after lyophilization. Unfortunately, this material proved to be very hygroscopic and in the presence of water gave a solution with a pH lower than 2, leading to hydrolysis of the carboxylate ester at the 5'-position of the ribofuranose ring. To enhance compound stability, the sodium salt (3) of acid 2 was prepared. Cleavage of the ester protecting group of 33 and purification by ion-exchange chromatography on Dowex 50W-X4 resin (Na^+) gave a 46% yield of the sodium carboxylate 3. This material also was hygroscopic but did not decompose on storage at room temperature because no carboxylic acid groups were available to catalyze the hydrolysis of the succinate ester in the presence of water.

The next method investigated for the introduction of a carboxylic acid-functionalized tether group onto ribavirin was the condensation of a keto ester with the 2',3'-hydroxyl groups of the ribofuranose ring of the drug. After removal of the ester protecting group from the resultant 2',3'-ketal, the free carboxylic acid group would be

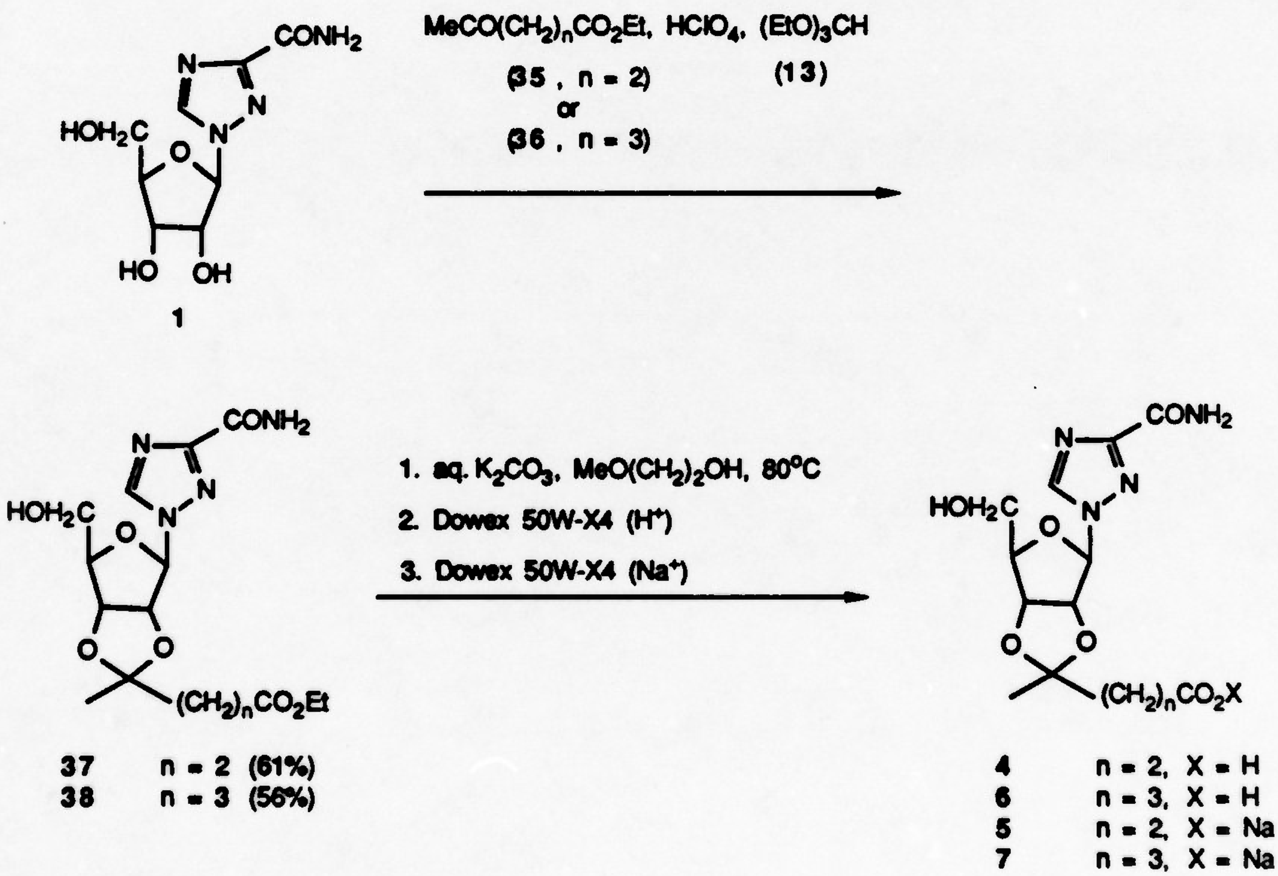
used to form the tether with MAbs. We had proposed that the ketal would be sufficiently stable under physiologic conditions (pH 7.2) to permit the MAb to deliver the drug to the virally infected cell, and that, once in the cell, lysozomal hydrolases would release the drug.

The two keto esters selected for ketalization with ribavirin were ethyl levulinate (35) and ethyl 4-acetylbutyrate (36). Ketalization of ribavirin with 35 failed under the standard ketalization conditions using p-toluenesulfonic acid as the acid catalyst in refluxing benzene because of the low solubility of ribavirin. The use of excess p-toluenesulfonic acid (10 equivalents) in ethyl levulinate as both the reactant and the solvent--a procedure that is effective at forming the acetonide of some nucleosides in acetone[15]--did not lead to ketalization. Bis(4-nitrophenyl) phosphate in either dimethyl sulfoxide or ethyl levulinate as the solvent also failed. Bis(4-nitrophenyl) phosphate was reported to be effective at forming the acetonide of uridine in acetone.[16]

The ketal 37 was successfully prepared using a catalytic amount (0.14 equivalent) of the much stronger acid, perchloric acid,[17] in ethyl levulinate (35) as the solvent, containing triethyl orthoformate (13) to remove the water formed in the reaction (Scheme I-5). Reaction for two hours at room temperature followed by chromatography on silica gel with 0-10% methanol/chloroform gave a 61% yield of ketal ester 37. Only one diastereoisomer was isolated in this reaction, in which the newly formed chiral center at the 2-position on the

dioxolane ring had an R configuration. This configuration was established from the Nuclear Overhauser Effect (NOE) between the 1'-proton on the ribofuranose ring and the β-methylene protons of the 2-propionate group of the dioxolane ring that was observed in the ^{1}H NMR spectrum of 37. The appearance of such an NOE indicates that the 1' and methylene protons are within 3.5 Å of one another, leading to the conclusion that the propionate side chain lies under the ribose ring and the methyl group projects outward.

Scheme I-5. Syntheses of Ribavirin 2',3'-Ketals and Their Sodium Salts



Ketalization with ethyl 4-acetylbutyrate (36) using perchloric acid as the catalyst afforded a 25% yield of a mixture of two diastereoisomeric ketals (38) after chromatography on silica gel with 0-10% methanol/chloroform. The integrals of the ethyl ester quartets at 4.05 and 4.01 ppm in the ^1H NMR spectrum of the ketal mixture were in a ratio of 4:1.

The hydrolysis of the ester protecting group on ketals 37 and 38 was investigated next. Aqueous potassium hydroxide in ethanol proved to be too basic and also led to hydrolysis of the carboxamide group at the 3-position of the triazole ring. This side reaction could be minimized by using potassium carbonate as the base. Methoxyethanol was employed as the organic solvent to improve the solubility of the starting material. Ketal esters 37 and 38 were treated with 3.0 equivalents of 1 M aqueous potassium carbonate in methoxyethanol (1:1/v:v) at 80°C for two hours. After cooling to room temperature, the reaction mixtures were partially neutralized with Dowex 50W-X4 resin (H^+, pH 5) before purification by chromatography on DEAE Sephadex A-25 (0.0-0.5 M ammonium bicarbonate gradient) and ion exchange with Dowex 50W-X4 resin (H^+) to give the ketal acids 4 and 6 in yields of 56% and 25%, respectively, as fluffy white solids.

4: ^1H NMR (dimethyl sulfoxide-d_6) δ 1.23 (s, 3 H, CH_3), 1.95 (m, 4 H, CH_2CH_2), 3.23 (m, 2 H, 5'-CH_2), 4.23 (m, 1 H, 4'-CH), 4.94 (br d, J = 6.1 Hz, 1 H, 3'-CH), 5.13 (br d, J = 6.1 Hz, 1 H, 2'-CH), 6.19 (br s, 1 H, 1'-CH), 7.68 (br s, 1 H, CONH), 7.89 (br s, 1 H, CONH), 8.83 (s, 1 H, 5-HetH).

6: ^{1}H NMR (deuteriomethanol-d_4) δ 1.33 (s, 2.4 H, CH_3), 1.50 (s, 0.6 H, CH_3), 1.60–1.75 (m, 0.8 H, $(\underline{CH_2})_2CH_2CO_2$), 1.75–1.90 (m, 3.2 H, $(\underline{CH_2})_2CH_2CO_2$), 2.20–2.40 (m, 2 H, CH_2CO_2), 3.55 (d, J = 1.3 Hz, 0.8 H, 5'-CHb), 3.58 (d, J = 1.3 Hz, 0.2 H, 5'-CHb), 3.62 (s, 1 H, 5'-CHa), 4.37 (dt, J = 1.8 Hz, J = 5.7 Hz, 1 H, 4'-CH), 4.92 (d, J = 1.8 Hz 0.2 H, 3'-CH), 4.99 (d, J = 1.8 Hz, 0.8 H, 3'-CH), 5.25 (d, J = 1.3 Hz, 0.8 H, 2'-CH), 5.32 (d, J = 1.3 Hz, 0.2 H, 2'-CH), 6.19 (d, J = 1.3 Hz, 1 H, 1'-CH), 8.68 (s, 1 H, 5-HetH).

Both acids were very hygroscopic and proved to be unstable in hydrated form, being hydrolyzed to ribavirin and the levulinic and acetylbutyric acids. Hydrolysis also occurred on dissolution in water (pH 4). However, the acids were quite stable in anhydrous solvents and in basic solution. Therefore, to prevent this hydrolysis problem, the sodium salts 5 and 7 of 4 and 6, respectively, were prepared and will be used for tethering. The salts were stable on storage at room temperature.

Because of hydrogen binding of the hydroxyl protons of these derivatives with the polar solvent in which the ^{1}H NMR spectra were run, and because the ketals 5 and 7 were diastereomeric mixtures at the 2-position of the dioxolane ring of the ketal, the interpretation of the spectral signals proved to be difficult. Nevertheless, the spectra indicated that esterification of the ribofuranose ring of ribavirin at the 5'-hydroxyl group shifted both the 4'- and 5'-protons

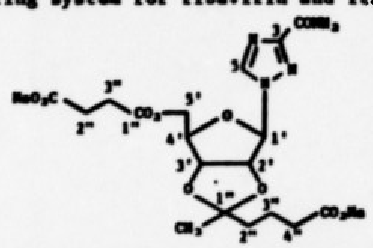
downfield and that ketal formation shifted the 1',2',3',4'-protons downfield, with the largest shift occurring for the 2',3'-protons. However, these 2'- and 3'-protons could not be distinguished. Decoupling experiments should aid in their identification. The signals for the protons on the methyl group at the 2-position of the dioxolane ring of the ketals 5 and 7 appeared as two singlets whose integrals corresponded to the ratio of the diastereomers (9:1 and 8:2, respectively) found by high-performance liquid chromatography (HPLC) analysis. In addition, the signal for the 5-hydrogen on the triazole ring of 5 also appeared as two singlets in a ratio of 8:2.

To help establish the structure of these derivatives, their ^{13}C NMR spectra were run and compared with that of ribavirin (Table I-1). The ^{13}C NMR spectrum of ribavirin in hexafluorobenzene has been reported.[18] The triazole ring and amide carbonyl carbon signals [146.3 (5), 158.4 (3), and 161.8 ppm ($CONH_2$)] were identified by comparison with the signals of the carbons of other ribavirin analogs. The corresponding signals for ribavirin were shifted downfield by approximately 8.7 ppm when dimethyl sulfoxide-d_6 was used as the solvent. On derivatization of the ribofuranose ring, the positions for these signals remained constant. Esterification of the 5'-hydroxyl group of ribavirin shifted the 5'-carbon signal 2.3 ppm upfield and the 4'-carbon signal 3.6 ppm downfield but had very little effect on the 2'- and 3'-carbon signals. The signal for the carboxylate carbon of 3 was assigned downfield relative to that of the ester carbonyl because similar downfield shifts of 2 ppm were observed

Table I-1. ^{13}C Spectra of Ribavirin (1) and Its 5'-Hemisuccinate Sodium Salt 3 and 2',3'-Ketal Sodium Salts 5 and 7[a].

	Carbon (ppm)[b]													
	Triazole Ring			Ribofuranose Ring					Tether Group					
Cmpd	3	5	$CONH_2$	1'	2'[c]	3'[c]	4'	5'	CH	1"	2"	3"	4"	CO
1	167.3	155.0	170.5	101.8	84.6	80.1	95.6	71.3						
3	167.4	155.4	170.5	101.8	84.2	80.4	92.0	73.7		183.3	40.8[c]	42.3[c]		185.9
5	167.4	155.4	170.4	102.8	94.0	91.5	98.3	71.4	33.4	124.5	30.7	48.3[c]	48.6[c]	187.2
				(94.4)	(92.0)	(98.5)		(34.2)	(124.9)	(31.4)				
7	167.3	155.3	170.4	102.9	94.0	91.5	98.3	71.3	33.4	124.5	42.5	46.2		186.8
					(91.8)			(33.7)	(124.9)	(43.3)				

[a]The general numbering system for ribavirin and its derivatives is:



[b]Relative to TMS. Observed signals for carbons of minor diastereomer appear in parentheses.

[c]Signals could be transposed.

for the carbonyl carbon on conversion of the carboxylic acids 4 and 6 to the corresponding sodium carboxylates 5 and 7. As in the spectrum of ribavirin, the 2'- and 3'-carbon signals could not be assigned. This was also the case with the methylene carbons adjacent to the carbonyl carbons of the tether group.

The 2'- and 3'-carbon signals in the spectra of ketals 5 and 7 were shifted downfield by approximately 10 ppm, whereas the 4'-carbon signal was shifted upfield by 2.7 ppm. The spectra of 5 and 7 clearly indicated that both were diastereomeric mixtures. Two signals were observed for the carbons at the 2-position of the dioxolane ring (1") of the ketal and the two adjacent carbons (CH_3 and 2"). In the spectrum of ketal 5, two signals were observed for the 2'-, 3'-, and 4'-carbons; in the spectrum of 7, only one of the two ribofuranosyl carbons making up the dioxolane ring had two distinct signals, whereas the other carbon and the 4'-carbon signals had downfield shoulders. The ratio of the heights of the signals for the diastereomeric carbons agreed with the diastereomeric ratios found by ^1H NMR spectroscopy and HPLC. The signals for the minor diastereomer were downfield by $\leq$ 0.8 ppm relative to those of the major diastereomer. These minor signals and their shift positions were useful in making assignments for the 2'-, 3'-, and 4'-carbons of the sugar and the 1"-, 2"-, and methyl group carbons on the tethers. Again, the 2'- and 3'-carbons of the ribofuranose ring of 5 and 7 and the 3"- and 4"-carbons on the tether of 5 could not be conclusively identified.

After the identity of 3, 5, and 7 had been confirmed by elemental and spectral analyses, these compounds were submitted to Utah State University for in vitro antiviral screening.

To accurately assess the activity of these derivatives in biological assays, their stability under the assay conditions had to be determined. If ribavirin were to be released by hydrolysis in the assay medium rather than in the virally infected cell, false positive results would be obtained. Therefore, 4.0-mM solutions of the sodium salts 3, 5, and 7 were incubated under physiologic conditions (0.05 M potassium phosphate, pH 7.4, 0.15 M NaCl; 37°C bath) and aliquots were removed and analyzed by reverse-phase HPLC. As shown in Table I-2, the reverse phase chromatography conditions cleanly separated the derivatives from ribavirin. HPLC analysis indicated that ribavirin and ketals 5 and 7 were stable after incubation at physiologic pH at 37°C for 15 days. The hemisuccinate 3 slowly hydrolyzed with 17% being converted to ribavirin after seven days and 30% after 15 days. A 5'-hemisuccinate with an alkyl substituent α to the ester bond would have a much slower rate of hydrolysis because of steric hindrance. We plan to prepare conjugates of this type.

Exploratory synthetic studies were begun to prepare another 5'-functionalized ribavirin derivative--the 5'-[3-(2-pyridyl)dithio-propyl] phosphate ester 45 (Scheme I-6), which could be reduced to 3-mercaptopropyl 5'-ribavirin phosphate (8). Phosphate ester 8 could

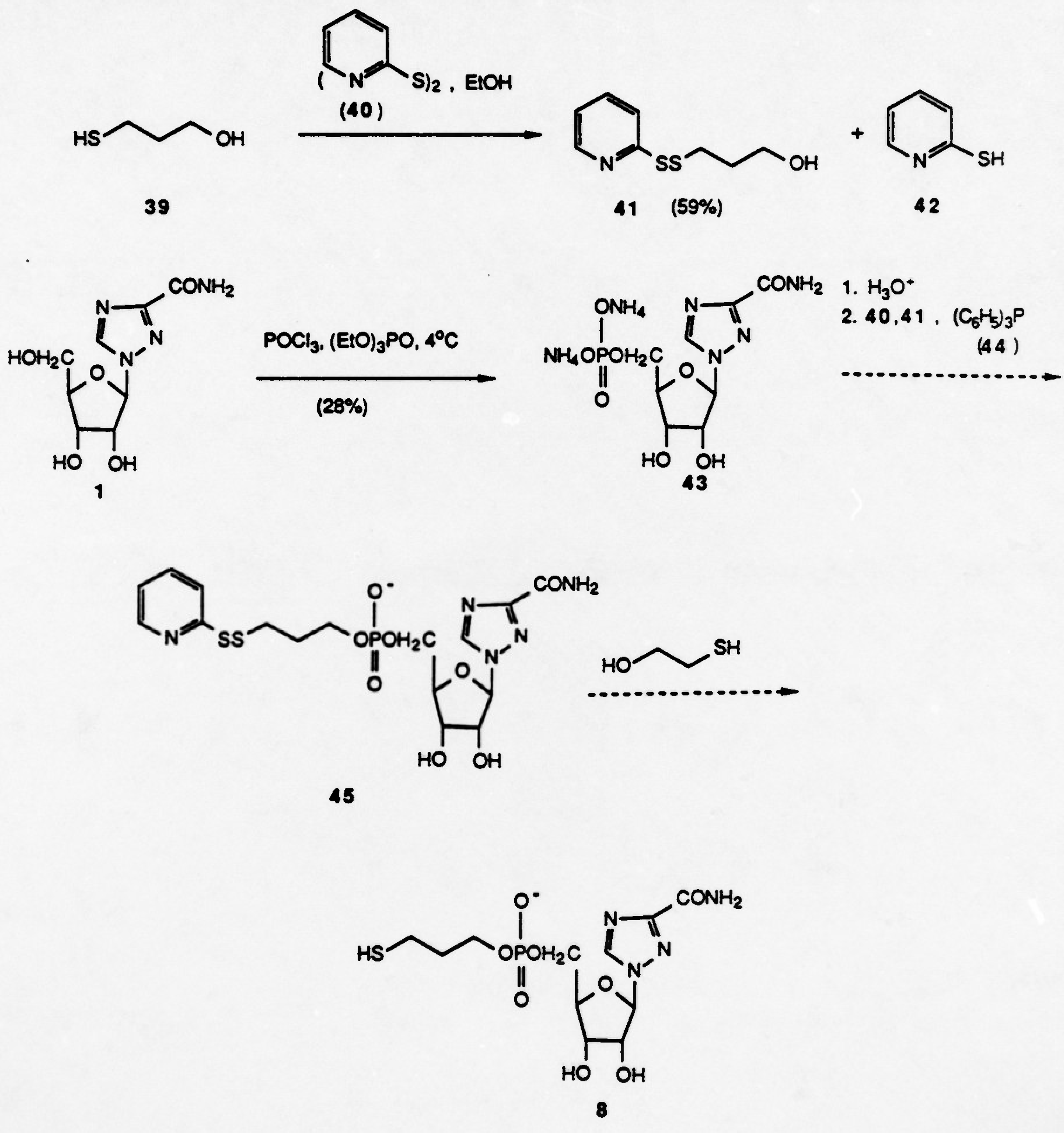
Table I-2. Reverse-Phase HPLC[a] of Ribavirin and Its Derivatives 3, 5, and 7.

Compound	Retention Time (min)
1	2.02
3	2.27
7	3.09 (4.82)[b]
5	6.58 (8.62)[b]

[a]C18 Nova-Pak column with elution at a flow rate of 2.0 ml/min with 98% 0.1 M sodium acetate/2% methanol (5 min), then a linear gradient to 80% 0.1 M sodium acetate/20% methanol (6 min), followed by a rinse with the original buffer mixture (1 min) and re-equilibration (4 min), and detection at 254 nm.

[b]Minor diastereomer is in parentheses. Ratio of peak areas was 9:1 for 5, 8:2 for 7.

Scheme I-6. Progress toward the Synthesis of a 5'-Phosphate Ester of Ribavirin



undergo disulfide exchange with monoclonal antibodies to form a disulfide bond between the 3-thiopropyl 5'-ribavirin phosphate ester mercapto group and a cysteine group formed by reduction of the monoclonal antibody. Intracellular disulfide exchange would release 3-mercaptopropyl-5'-ribavirin phosphate from the conjugate in vivo. Attack of 8 by phosphatases would afford ribavirin. This type of disulfide interchange is used to deliver ricin in ricin-monoclonal antibody conjugates.[19]

The starting material for this synthesis, 3-mercaptopropanol (39) was treated with 2,2'-dipyridyl disulfide (40) in ethanol for four days according to a literature procedure.[9] The mixed disulfide product (41) was isolated in 59% yield by flash column chromatography of the concentrated reaction mixture on silica gel with 25-67% ethyl acetate/hexanes.

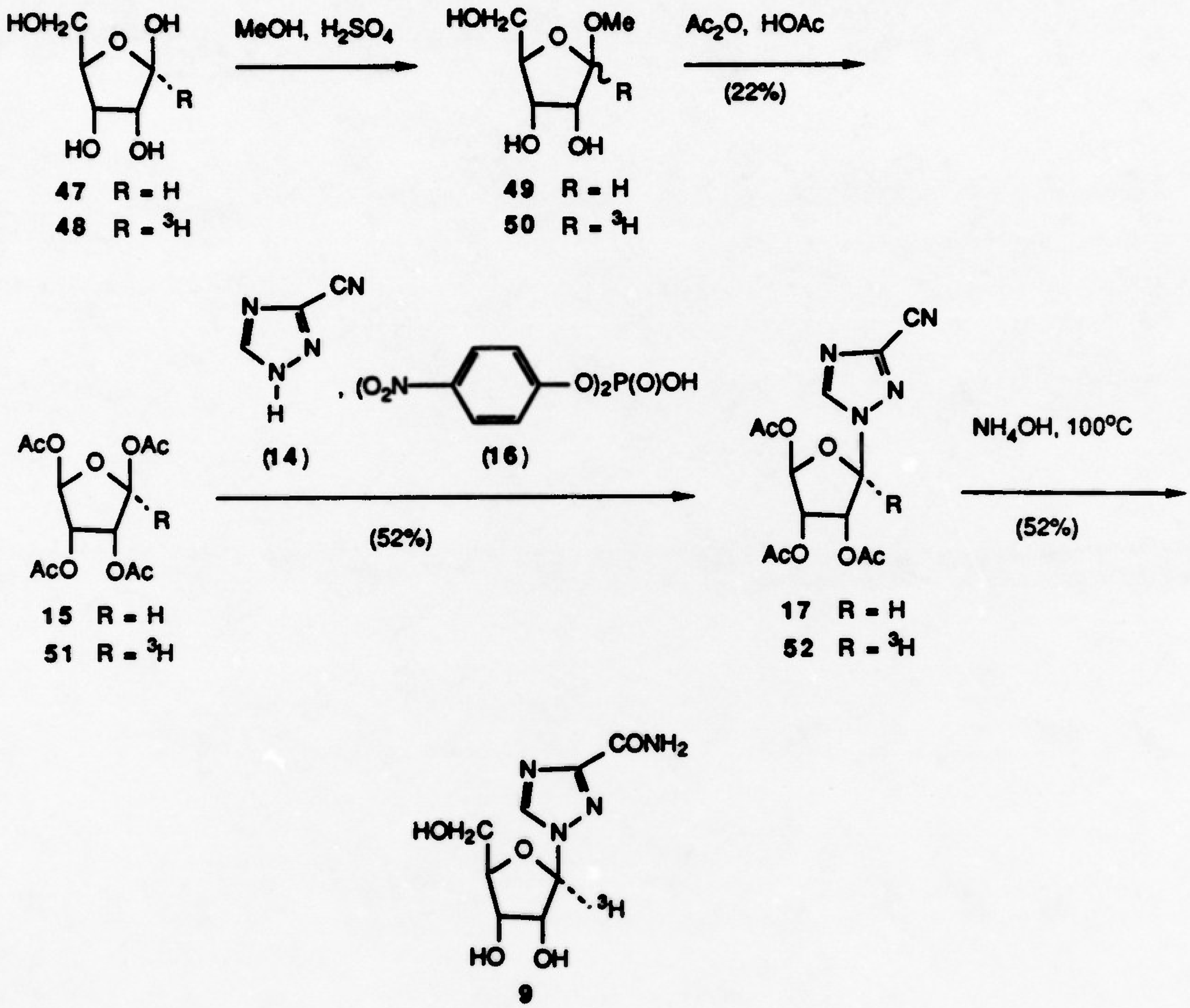
The other target intermediate in the convergent synthesis of phosphate diester 8 was ribavirin 5'-phosphate, which was prepared by reaction of ribavirin with phosphorous oxychloride and isolated in 28% yield as the diammonium salt (43) by DEAE Sephadex A-25 chromatography using a 0.0-0.5 M ammonium bicarbonate gradient followed by lyophilization. Coupling of 43 with alcohol 41 and deprotection of the product 45 should afford 8. Coupling of 1 and 39 using activated cyclic phosphate esters will also be explored as a route to 45.

During the first annual reporting period of this contract, work was also completed on the synthesis of [1'-^{3}H]ribavirin (9) from [1-^{3}H]D-ribose (Scheme I-7). The specific activity of 9 should be sufficient to establish loading levels of the ribavirin derivatives on MAbs. Two cold runs on the scale that would be used for the radiolabelled synthesis were first performed to verify the experimental methods. [1'-^{3}H]Ribavirin of 97.5% radiochemical purity (specific activity 0.82 mCi/mmol) was obtained (Figure I-1).

D. Discussion and Conclusions

In vitro testing results have established that the 5'-hemisuccinate ester of ribavirin has *in vitro* antiviral activity but that the ketals 5 and 7 are inactive. Before conclusions can be drawn about the suitability of these compounds as tethering candidates, the conjugates of these drug derivatives with MAbs will have to be prepared and screened. The hemisuccinate may very well hydrolyze at physiologic pH; however, this may not interfere with a sufficiently rapid delivery of the drug-MAb conjugate to the surface of the virally infected cell. Hydrolytic release of the drug at the cell surface would afford a high local concentration of the drug. The lack of biological activity of the ketal derivatives of ribavirin may be caused either by their inability to enter the virally infected cell or by their stability in the cell. Only by tethering these derivatives to MAbs and conducting antiviral screening can these questions be answered. If the hemisuccinate ester proves to be too labile an ester for use in targeted delivery, other ester tethering linkages that are

Scheme I-7. Synthesis of [1'-^{3}H]Ribavirin



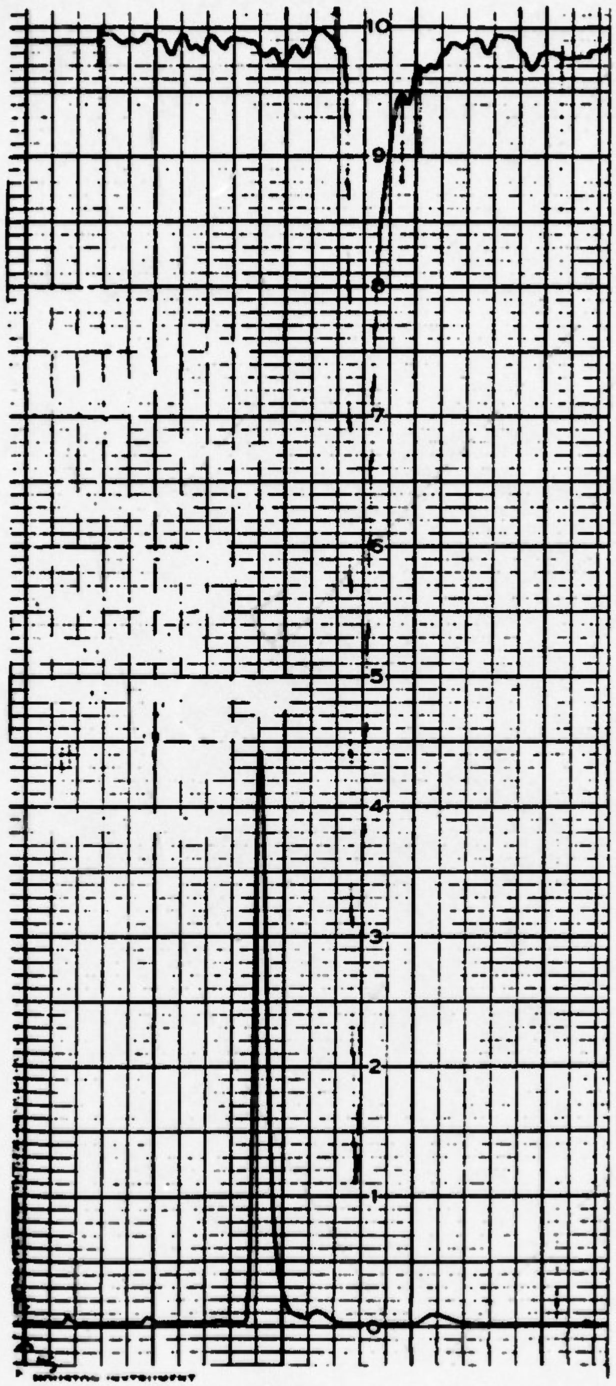


Figure I-1. UV and Radiochemical Chromatograms of [1'-^{3}H]Ribavirin.

more sterically hindered to hydrolysis will be prepared. If the ketal tether is not hydrolyzed by lysozomal hydrolytic enzymes, more labile acetal linkages will be explored.

A major thrust of the synthetic effort in the next reporting period will be directed at the phosphate diesters. By manipulation of the functional group on the alkyl phosphate ester at the 5'-position, linkages to the lysine ε-amino groups or the mercapto groups of reduced MAbs can be achieved.

E. References

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II. In Vitro Culture of Pichinde Virus

A. Introduction

To achieve the goals of this project, it was necessary to obtain the virus to be used in the project, to ensure that the virus was not contaminated with other viruses or other organisms, to build pools that were sufficiently infective in the *in vitro* and *in vivo* systems, and to quantify those pools. The assurance that the virus was not contaminated was important because the virus to be used in our program was provided initially by the USAMRIID and had been used in that facility in a laboratory where other, potentially more infectious, viruses had been used. This assurance of virus purity was achieved by twice isolating the virus from single plaques and building a virus pool from those plaques. Identification of the virus was confirmed by immunofluorescence using a specific antibody.

B. Materials and Methods

Viruses. Strain An4763 of Pichinde virus was received as a 12th-passage guinea pig spleen homogenate from Dr. Joseph D. Gangemi of the University of South Carolina School of Medicine. Dr. Gangemi originally obtained the virus from Dr. Peter Jahrling of USAMRIID, who had adapted it to cause lethal infections in guinea pigs. Strain CoAn3739 of Pichinde virus was obtained from the American Type Culture Collection (ATCC, Rockville, MD). When received at Utah State University, both viruses were grown one passage in Vero 76 cells and then were twice

plaque-purified in the same cell line. The twice plaque-purified viruses were used to prepare the virus pools that will serve as inocula for *in vitro* and *in vivo* studies. Twenty T75 flasks of Vero 76 cells were inoculated with virus at a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU)/cell. After three days incubation at 37° C, the cells were frozen and thawed three times, then centrifuged at 1900 rpm for 10 minutes. The cell-free supernates were pooled, dispersed to ampules, and stored at -80°C. The viruses were found to be free of mycoplasma contamination and their identity was confirmed by immunofluorescence test using specific antisera.

Cells. African green monkey kidney (Vero 76) cells were obtained from ATCC. They were grown in minimum essential medium (MEM), obtained from Grand Island Biological Laboratories (GIBCO, Grand Island, NY), with 5% fetal bovine serum (FBS) and 0.1% $NaHCO_3$.

Mycoplasma Testing. All cell cultures used were periodically tested for presence of mycoplasma by the MycoTect system (GIBCO) as directed in the product-use instructions. Indicator cell cultures were used in each test for negative and positive controls. The positive control wells were inoculated with ATCC 29052, *Mycoplasma hyorhinis*, and with ATCC 25528, *Mycoplasma arginini*.

Immunofluorescence Test. A direct immunofluorescent test was used to confirm the identity of our Pichinde virus preparations. In this test, 15-mm coverslips (3/petri dish) covered by a monolayer of Vero 76 cells were exposed to the virus diluted in MEM without FBS. After a 1-hour adsorption at 37°C, MEM without FBS was added to the plate. The

cells were then incubated for 22 to 24 hours, and fixed with cold 80% acetone for 10 minutes. They were then stained for 24 hours with 100 µl of specific anti-Pichinde virus guinea pig antiserum conjugated to fluorescein isothiocyanate. The cells were then rinsed twice in distilled water, a drop of elvanol mounting medium was added, and the cells were covered with a coverslip. The fluorescence was read using a Zeiss epifluorescence microscope at 160X magnification. The guinea pig Pichinde virus antiserum-fluorescein conjugate (conjugate #234, 4/84) was provided by Dr. Meir Kende of USAMRIID.

Indirect Immunofluorescence Assay. The Pichinde virus used in our studies was quantified using an indirect immunofluorescence cell count (IFCC) assay, described in more detail in Section X. In this procedure, 24-well tissue culture plates, 96-well microplates, or 15-mm coverslips were used. The same procedure as described above for direct immunofluorescence was used except that the cells were fixed for 5 minutes with cold 0.025% glutaraldehyde in phosphate-buffered saline (PBS) and rinsed three times with PBS containing 100 mM glycine, and the monolayer was exposed to hyperimmune (anti-Pichinde virus) mouse serum for 1 hour. The monolayer was then exposed to a second antibody consisting of goat anti-mouse gamma globulin labeled with fluorescein (Lot 8F, Antibodies, Inc., Davis, CA). This stain was also removed and the cells were rinsed twice and examined for fluorescence. Each assay included uninfected cell control wells for each sample.

Fluorescent Antibody Conjugates. Five different commercial preparations of fluorescein isothiocyanate (FITC) conjugated to anti-

mouse antibody were compared. They were Ab 1: Goat anti-mouse gamma globulins, fluorescein-labeled, Lot No. 8F, Antibodies, Inc., Davis, CA; Ab 2: Rabbit anti-mouse, Cat. No. CL 6002-F, Cedarline, Hornby, Ontario, Canada; Ab 3: Fluorobody goat antibody to mouse IgG (H+L), Cat. No. 8608-13, Lot AC024, Bionetics, Kensington, MD; Ab 4: Goat antibody to mouse IgG (H+L) affinity purified, Cat. No. 401214, Lot No. 202306, CalBiochem, La Jolla, CA; Ab 5: FITC anti-mouse IgG (H+L), Cat. No. 51061A, Lot No. MDF006, HyClone Laboratories, Logan, UT.

Plaque Assay Procedure. Each pool of Pichinde virus was also titrated in Vero 76 cells using plaque formation. Disposable 24-well tissue culture plates were seeded with 5 x 10^5 Vero 76 cells/ml, and 18 hours later the cell monolayer was drained and rinsed. Varying dilutions of the Pichinde virus to be titered, suspended in MEM containing 2% FBS and 50 μg/ml of gentamicin, were added to the cells and allowed to adsorb for 45 minutes, with rocking of the plate every 5 minutes. The plate was then overlaid with MEM, containing 2% FBS and either 1% methylcellulose or 0.75% Noble agar, and incubated at 37°C for 4 days. After this incubation period, 0.5 ml of neutral red dye (0.1 mg/ml in PBS) was added to the overlay. After an additional 4 hours of incubation, the plaques were counted. When the plaques were not to be picked from the plate for use in further studies, the overlay was stained with crystal violet rather than neutral red since the crystal violet dye makes the plaques more distinctly visible. In this procedure, the cells were fixed with 10% formalin in PBS for 24 hours at room temperature, then poured from the plate, and the plate was tapped to remove the agarose layer. A 1.0-ml aliquot of 0.2% crystal violet was added to the remaining fixed cells

in each well and kept on the cells for 1 hour. This stain was removed by decanting and rinsing the cells with distilled water. The plaques were then counted.

C. Results and Discussion

Both strains of virus prepared in our laboratory were confirmed to be Pichinde virus by immunofluorescence. Both produced readily discernible plaques in Vero 76 cells, although with the higher-titered virus pools, less dilute virus levels often failed to produce uniform plaques (Table II-1). We presume that this was a result of defective interfering particles, as has been reported by others.[1,2,3]

Both virus strains also caused distinct immunofluorescence in cells using the indirect immunofluorescence assay. In this assay, we ran a comparison experiment using five different commercial preparations of FITC conjugated to anti-mouse antibody. The results are summarized in Table II-2. Nonspecific staining was seen, particularly with Ab 2 (Cedarline). The Ab 1 (Antibodies, Inc.) gave the most selective staining and was therefore selected as the "second" antibody in the indirect immunofluorescent test.

The specificity of the indirect immunofluorescent assay was confirmed by comparing IFCC obtained with our hyperimmune mouse serum with those counts obtained using FITC-conjugated guinea pig anti-Pichinde virus antibody obtained from USAMRIID. The cell counts were virtually identical. Both immunofluorescence procedures resulted in bright,

distinct staining of Vero 76 cells infected with plaque-purified Pichinde virus (Table II-3).

The pool of An4763 Pichinde virus strain prepared for our studies had a titer of 4.0×10^5 plaque-forming units (PFU)/ml and 1.6×10^6 IFCC/ml. The CoAn3739 Pichinde virus pool had a titer of 1.7×10^7 PFU/ml and 2.6×10^8 IFCC/ml.

D. Conclusions

Large pools of two strains of twice plaque-purified Pichinde virus have been prepared. Both pools were quantified in Vero 76 cells by plaque formation and by immunofluorescent cell count, the latter using an indirect immunofluorescent assay that we have developed. Some indication of defective interfering particles was often seen in our titrations when high concentrations of virus failed to produce uniform plaques.

E. Literature Cited

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3. Stanwick, T. L. and Kirk, B. E. 1976. Analysis of baby hamster kidney cells persistently infected with lymphocytic choriomeningitis virus. J. Gen. Virol., 32:361-367.

Table II-1. Titration of Two Strains of Pichinde Virus in Vero 76 Cells Using Plaque Formation Endpoint.

Dilution of Pool	Strain An4763	Strain CoAn3739
10^{-1}	No visible plaques	No visible plaques
10^{-2}	TNTC*	TNTC
10^{-3}	55, 41	TNTC
10^{-4}	3, 5	TNTC
10^{-5}	0, 0	61, 72
10^{-6}	0, 0	0, 0
Titer:	1×10^5 PFU/ml	1.7×10^7 PFU/ml

*Too numerous to count.

Table II-2. Comparison of Commercial FITC-Anti-Mouse Antibody Conjugates for Efficacy in Indirect Fluorescence Antibody Testing for Antibody to Pichinde Virus.[a]

Antibody[b]	Dilution Antibody	Nonspecific Reaction[c]	Specific Reaction[d]
Ab 1	1:100	-	++++
Ab 1	1:200	-	+++
Ab 1	1:400	-	++
Ab 1	1:800	-	+
Ab 2	1:100	++ (debris)	++ (debris, difficult to read)
Ab 2	1:200	+ (some debris)	- (too weak to read)
Ab 2	1:400	+ (some debris)	- (too weak to read)
Ab 2	1:800	-	-
Ab 3	1:100	-	± (too weak to read)
Ab 3	1:200	-	-
Ab 3	1:400	-	-
Ab 3	1:800	-	-
Ab 4	1:100	-	± (too weak to read)
Ab 4	1:200	-	-
Ab 4	1:400	-	-
Ab 4	1:800	-	-
Ab 5	1:100	±	++
Ab 5	1:200	-	+
Ab 5	1:400	-	±
Ab 5	1:800	-	-

[a] The procedure was described under Indirect Immunofluorescence Assay except that the second antibody was varied.

[b] Ab 1: Goat anti-mouse gamma globulin, fluorescein-labeled, Lot. No. 8F, Antibodies Incorporated, Davis, CA.

Ab 2: Rabbit anti-mouse, Cat. No. CL 6002-F, Cedarline, Hornby, Ontario, Canada.

Ab 3: Fluorobody goat antibody to mouse IgG (H+L), Cat. No. 8608-13, Lot No. AC024, Bionetics, Kensington, MD.

Ab 4: Goat antibody to mouse IgG (H+L) affinity purified, Cat. No. 401214, Lot No. 202306, CalBiochem, La Jolla, CA.

Ab 5: FITC anti-mouse IgG (H+L), Cat. No. 51061A, Lot No. MDF006, HyClone, Logan, UT.

[c] Staining of uninfected control cells observed.

[d] Staining of infected cells observed.

Table II-3. Comparison of Direct and Indirect Immunofluorescence Staining of Pichinde Virus-Infected Vero 76 Cells.

Type of Staining	Nonspecific Reaction[a]	Specific Reaction[b]
Direct staining with guinea pig anti-Pichinde antibody	-/+	++
Indirect staining with hyperimmune mouse serum and Antibodies Incorporated goat anti-mouse antibody	-/+	++
Indirect staining with hyperimmune mouse serum and Boehringer goat anti-mouse antibody	-	++++

[a] Nonspecific reaction scored from - (no specific staining visible) to ++++ (severe nonspecific staining overwhelming any specific staining).

[b] Specific reaction scored from - (no visible specific staining) to ++++ (the most intense specific staining resulting from any hyperimmune sera tested, easily visible).

III. Production of Large Pools of Pichinde Virus

A. Introduction

Large pools of Pichinde virus were prepared for use as antigens in the characterization of monoclonal antibodies. Pichinde virus strains CoAn3739 and An4763 were grown in roller bottle cultures of Vero cells. The resulting CoAn3739 virus pool had a titer of 3×10^7 fluorescent cell forming units (FCFU)/ml and the An4763 virus pool had a titer of 4×10^6 FCFU/ml. In one set of experiments, the effect of freezing and thawing on Pichinde virus infectivity was examined. The first step in harvesting many viruses from infectious cell cultures is a series of freezings and thawings. This removes the cells from the walls of the culture vessel and causes cell membranes to be disrupted, thus releasing intracellular virus. In the case of Pichinde virus, the virus must bud out through the cytoplasmic membrane and thereby acquire an envelope in order to mature to an infectious virus, so freezing and thawing would not be expected to increase the infectious titer of harvested cell culture fluids. However, for our purposes (that is to generate Pichinde virus antigens for immunological testing) freezing and thawing might be beneficial as long as the process does not decrease infectious Pichinde virus concentrations.

B. Materials and Methods

Cells. Virus production and assays were performed with the Vero 76 line of African green monkey kidney cells obtained from the ATCC. Vero cells were cultured in MEM (GIBCO) containing 9% FBS (HyClone Labs).

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC) and the CoAn3739 strain of Pichinde virus (obtained from the ATCC) were used in these studies. Pichinde virus used as the seed virus in virus production was twice plaque-purified as described in Section II of this report.

Production of Pichinde Virus in Roller Bottle Culture. Roller bottle cultures of Vero cells were inoculated with plaque-purified Pichinde virus at a multiplicity of infection (MOI) of 0.5 to 2 FCFU per cell. Virus was allowed to adsorb to cells for 1 hour, then 75 ml of MEM containing 2% FBS was added to each roller bottle. Infection was allowed to proceed for 4 days at 37°C. Virus was then harvested with one cycle of freeze/thaw and infectious cell culture fluids were stored at -15°C.

Virus Assays. Pichinde virus assays were by an indirect immunofluorescence assay described in Section X of this report. The primary antisera was hyperimmune anti-Pichinde virus sera prepared in adult C57Bl/6 mice as described in Section IX of this report. The second antibody in the indirect immunofluorescence assay was affinity-purified goat anti-mouse IgG fluorescein conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN).

C. Results and Discussion

The Pichinde virus-infected roller bottle cultures showed extensive cell degeneration 4 days after inoculation with either strain of virus.

The culture fluids were turbid and most of the cells were detached from the vessel surface. There was no indication of yeast, bacterial, or fungal contamination. The cytopathic effect appeared to be a direct result of virus infection.

A 750-ml pool of Pichinde virus strain An4763 was produced. The concentration of infectious Pichinde virus in the An4763 was determined to be 4 x 10^6 FCFU/ml. The pool of Pichinde virus strain CoAn3739 consisted of 300 ml of cell culture fluids with a Pichinde virus concentration of 3 x 10^7 FCFU/ml. Over the past year, each time Pichinde virus pools have been produced, the CoAn3739 has yielded higher virus concentrations than the An4763 strain.

The effect of freezing and thawing on Pichinde virus infectivity was examined by freezing 50 ml of infectious strain An4763 cell culture fluids at $-15^{\circ}C$ and then thawing the fluids in a $20^{\circ}C$ water bath 20 hours later. A small sample of the fluids was removed for assay and the cycle was repeated. Prior to any freeze/thaw, the titer was 4 x 10^6 FCFU/ml, after three cycles the titer was 5 x 10^6 FCFU/ml, and after five cycles the titer was again measured at 4 x 10^6 FCFU/ml.

D. Conclusions

The CoAn3739 strain of Pichinde virus consistently produces 5- to 50-fold higher virus titers than does the An4763 strain. If it is determined that these two strains can be used interchangeably for monoclonal antibody characterization studies, such as Western blot analysis, then the use of CoAn3739 would offer an advantage.

Up to five cycles of freezing and thawing neither increases nor decreases the infectivity titers of harvested Pichinde virus-infected cell culture fluids.

IV. Development of Pichinde Virus In Vitro Models

A. Introduction

Initial studies with Pichinde virus described in Section II indicated that although the virus produced acceptable plaques, it did not readily induce cytopathic effect (CPE) in the Vero 76 cells used. Since the plaque-reduction parameter utilizes much larger amounts of test compound, the present studies were run in an attempt to develop a more acceptable CPE with this virus.

B. Materials and Methods

Virus. The An4763 and CoAn3739 strains of Pichinde virus, described in Section II, were used.

Cells. The following cells were used in a cell comparison study [the ATCC numbers indicate the American Type Culture Collection (Rockville, MD) reference number of the cell line indicated]: KB (ATCC CCL 17, human oral epidermoid carcinoma with HeLa Markers); BHK-21 (ATCC CCL 10, kidney cells derived from one-day-old Syrian golden hamsters); MDBK (ATCC CCL 22, Madin Darbey bovine kidney cells); L929 (ATCC CCL 1, NCTC clone 929, mouse connective tissue); Vero 76 (ATCC CRL 1587, African green Monkey kidney); MDCK (ATCC CCL 34, Madin Darbey canine kidney cells); MRC-5 (ATCC CCL 171, fetal male human lung diploid cells); WI-38 (ATCC CCL 75, fetal female human lung diploid cells); LLC-MK_2D (ATCC CCL 7.1, rhesus monkey kidney); MA-104 (embryonic rhesus monkey kidney cells, passage 52 from Dr. Mary K. Estes,

Baylor University, Houston, Texas). The cells were maintained in MEM with 10% FBS and 0.1% $NaHCO_3$, without antibiotics.

Procedure for Determining Influence of Cell Line on Production of Viral CPE. Each indicated cell line was seeded to 96-well disposable microtiter plates and incubated at 37°C until a uniform monolayer had formed. The medium was drained from the plates and varying dilutions of each Pichinde virus strain in medium (0.2 ml/cup) were added to triplicate cups in the plate; the medium used was MEM with 1% FBS, 0.18% $NaHCO_3$ and 50 µg/ml of gentamicin. The plates were sealed with plastic wrap and incubated at 37 °C for up to 14 days. On Day 6, the medium was aspirated and fresh medium with virus was added to the cell layer. Each plate was examined microscopically for visible CPE daily through the duration of the study.

Procedure for Determining the Influence of Cell Density on Production of Viral CPE. Three disposable 96-well microplates were seeded with 1, 2, 4, 8, 16, 32, 64, or 128 x 10^3 Vero cells/well; one row of 12 wells in each plate was used for each cell inoculum. The cell-suspending medium was MEM with 9% FBS and 0.1% $NaHCO_3$. After 37° C incubation for 24 hours, the degree of confluency was noted and one well at each cell density was exposed to CoAn3739 Pichinde virus at a dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, or 10^{-9}. Three wells at each cell density were not exposed to virus to serve as cell controls. The virus-suspending medium was MEM with 1% FBS, 0.18% $NaHCO_3$, and 50 µg/ml of gentamicin. The plates were examined microscopicslly for CPE production through 15 days post-virus exposure, when the normal control cells began to deteriorate.

Procedure for Determining the Influence of Trypsin on Production of Viral CPE. An 18-hour monolayer of Vero 76 cells was drained of medium and strain CoAn3739 of Pichinde virus at tenfold dilutions of 10^{-1} through 10^{-7}, suspended in MEM, containing 1 µg/ml of trypsin, 0.18% $NaHCO_3$, and either 0, 2%, or 9% FBS, was added (0.2 ml/cup) to triplicate cups in 96-well disposable microplates. The plates were sealed with plastic wrap and incubated at 37°C for up to 10 days. Each plate was examined microscopically daily for signs of viral CPE.

C. Results and Discussion

Effects of Cell Line. The results of this study are summarized in Table IV-1. Slight (0.5 to 1+) CPE was seen in BHK, MDBK, and MA104 cells at virus dilutions no higher than 10^{-3}. The CPE was slightly more extensive (1 to 1.5+) in Vero 76 cells, seen at dilutions from 10^{-1} through 10^{-4}. No CPE was discernible prior to 10 to 12 days post-virus exposure. The CPE was exhibited primarily as cell rounding. The effects of CPE seen in the Vero 76 cells was considered marginally acceptable for antiviral studies and these cells were used in a series of antiviral tests described in Section V of this report. Because of the marginality of the CPE demonstrated, further studies were run in an attempt to improve the CPE reading, as described further in this section.

We understand that Dr. Peter Jahrling of the USAMRIID has reported finding a satisfactory CPE using MDCK cells. In all cases in our experiments, these cells have failed to yield a visible CPE when infected with either the An4763 or the CoAn3739 strain of Pichinde virus. We have

no explanation for this anomaly unless the USAMRIID cells had a mycoplasma contaminant that exerted an additive effect on the viral CPE produced. Also, in view of the results seen in the next portion of this section, the cell density used by Dr. Jahrling may have varied from what we used, which may have enhanced the CPE reading seen in his laboratory. Details of media, serum, etc. used at USAMRIID have not yet been determined. All the cells used in our studies have been repeatedly assayed for mycoplasma according to the methodology described in Section II of this report and have been found free of this contaminant. We understand that investigators at Southern Research Institute (Birmingham, AL), who are also studying Pichinde virus *in vitro*, have had similar difficulties in obtaining acceptable CPE readings (Dr. William Shannon, personal communication).

Effects of Cell Density. It was thought that perhaps a lower initial cell density would prolong the life of the cell monolayer, allowing it to be in better condition when the Pichinde virus infection finally began to produce CPE. In this study, eight twofold dilutions of Vero 76 cells were tested for their ability to develop CPE when infected with nine tenfold dilutions of virus. The results at two observation times, Days 8 and 15, are summarized in Tables IV-2 and IV-3, respectively. The maximum CPE seen was definitely enhanced in this study by using lower cell densities, although it did not develop until approximately eight days after virus exposure. Much cell-rounding was seen, and in some cases the cell sheet was destroyed by the virus infection. At higher virus concentrations, no CPE was seen, again indicating the inhibitory effect of defective viral particles as described in Section II. The CPE

read at Day 15 was somewhat masked by deterioration of the cells, as evidenced by the cell controls run in the same plate. In two individual panel cups, complete destruction of the cell monolayer occurred. This may have been due to viral infection, but the isolated and rather sporadic nature of the occurrence makes these latter observations somewhat questionable.

Effect of Trypsin Treatment. We have found that inclusion of small quantities of trypsin in the test medium has an enhancing effect on the CPE produced by influenza[1] and rota[2] viruses. It was therefore thought that inclusion of this enzyme with the medium on Vero 76 cells exposed to Pichinde virus may have a similar CPE-enhancing effect. In the experiment, a barely confluent monolayer of cells in a 96-well microplate was exposed to seven tenfold dilutions of Pichinde virus suspended in MEM with 20 μg/ml of trypsin, 2 μg/ml of EDTA, 50 μg/ml of gentamicin, and 2% or 9% FBS. The plates were held for 10 days with microscopic examination being done daily. No enhancement of CPE was seen; the maximum was approximately 1+. This medium, which is used routinely for our influenza virus experiments in MDCK cells, was somewhat toxic to the Vero 76 cells. Further studies on the influence of trypsin on Pichinde virus infection, using lower concentrations of the enzyme, are to be run.

D. Conclusions

Vero 76 cells were most efficacious for developing Pichinde virus-infected CPE of the 12 cell lines studied. The CPE, however, was relatively slight (less than 2+, with 4+ being maximum) and required approximately 12 days to develop. Varying the initial Vero 76 cell

density exposed to the virus affected the time and degree of CPE produced, with initial cell inocula of 8 to 64 x 10^3 cells per well in 96-well microplates being most suitable. Again, the CPE was not pronounced except in a scattered and seemingly random pattern, but was seen as early as eight days post-virus exposure. High virus concentrations tended to inhibit CPE, suggesting the presence of defective interfering particles in the virus pools used. Trypsin (20 μg/ml) incorporated into the medium did not enhance viral CPE and appeared slightly toxic to the Vero 76 cells used. Since this enzyme has enhanced influenza and rota viral CPE in our hands, further studies of it, using Pichinde virus, will be undertaken.

E. Literature Cited

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Table IV-1. Cells Studied for Sensitivity to Pichinde Virus Using Production of Cytopathic Effect (CPE) as the Parameter of Infection.

Cell Line[a]	Level of CPE[b]	Virus Dilution[c]
KB	-	-
BHK	±	1:10, 1:100, 1:1000
MDBK	±	1:10, 1:100
L	-	-
L929	-	-
Vero	+	1:10 thru 1:10,000
MDCK	-	-
MRC-5	-	-
W1-38	-	-
LLC-MK_2D	-	-
HaK	-	-
MA-104	±	1:10, 1:100, 1:1000

[a] KB (ATCC CCL 17, human oral epidermoid carcinoma, HeLa markers); BHK (BHK-21, ATCC CCL 10, kidney cells derived from one-day-old Syrian hamsters); MDBK (ATCC CCL 22, Madin Darbey bovine kidney cells); L (L929 cells, in growth media containing 10% fetal bovine serum rather than 10% horse serum); L929 (ATCC CCL 1, NCTC clone 929, mouse connective tissue cell line grown in media supplemented with 10% horse serum); Vero (Vero 76, ATCC CRL 1587, African green monkey kidney); MDCK (ATCC CCL 34, Madin Darbey canine kidney cells); MRC-5 (ATCC CCL 171, fetal male human lung diploid cells); W1-38 (ATCC CCL 75, fetal female human lung diploid cells); LLC-MK_2D (ATCC 7.1, rhesus monkey kidney); HaK (ATCC CCL 15, adult Syrian hamster kidney cells); MA-104 (embryonic rhesus monkey kidney cells, passage 52 from Dr. Mary Estes, Baylor University, Houston, TX).

[b] - = no CPE; ± = slight cell-rounding; + = visible CPE.

[c] Virus dilution where CPE observed.

Table IV-2. Effect of Vero 76 Cell Density on CPE Production by Pichinde Virus Eight Days Post-Virus Exposure.

Cell Density ($\times 10^3$/well)	Viral CPE[a] at Virus Dilutions Indicated 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Cell Controls[b]
128	0	0	0	0	0	0	0	0	0	0 (120%)
64	0	0	0	1	1.5	1.5	1.5	0	0	0 (110%)
32	0	0	0	1.5	1.5	1.5	0	0	0	0 (100%)
16	0	0	0.5	1.5	1.5	1.5	4	0	0	0 (100%)
8	0	0	0.5	0.5	1	1	0	1	0	0 (50%)
4	0	0	0	0.5	1	4	1	1	0	0 (25%)
2	0	0	0	1	1	1	1	0	0	0 (10%)
1	0	0	0.5	0.5	1	1	1	0	0	0 (5%)

[a] Maximum CPE seen in a total of three plates run in parallel. Viral CPE scored from 0 (normal cells) to 4 (complete destruction of the cell layer).

[b] Cells exposed to sterile virus diluent only. Three cups used per cell density. ( ) = % of confluency at time of virus exposure.

Table IV-3. Effect of Vero 76 Cell Density on CPE Production by Pichinde Virus 15 Days Post-Virus Exposure.

Cell Density	Viral CPE[a] at Virus Dilutions Indicated									
($x\ 10^3$/well)	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Cell Controls[b]
128	0.5	0.5	0.5	1	1	1	0.5	0.5	0.5	0.5
64	0.5	0.5	0.5	1.5	1.5	1.5	1.5	0.5	0.5	0.5
32	0.5	0.5	0.5	1	1.5	1.5	0.5	0.5	0.5	0.5
16	0.5	0.5	0.5	1	1.5	2	4	0.5	0.5	0.5
8	0	0	0.5	1	1.5	1	0.5	1	0	0
4	0	0.5	0.5	1	1.5	4	1	0.5	0	0
2	3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4
1	3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4	3-4

[a] Maximum CPE seen in a total of three plates run in parallel. Viral CPE scored from 0 (normal cells) to 4 (complete destruction of the cell layer).

[b] Cells exposed to sterile virus diluent only.

V. *In Vitro* Antiviral Assay of Compounds Prepared by SRI International

A. Introduction

Prior to conjugating an antiviral compound to a monoclonal antibody (MAb) against Pichinde virus, it is important to determine whether the compound will exert a significant inhibitory effect on the virus. This is especially significant since the conjugation process involves the incorporation of certain chemical groupings onto a known antiviral compound. This report describes initial *in vitro* antiviral studies with compounds prepared by SRI International chemists as potential materials for the compound-MAb conjugations.

Two of the compounds were submitted quite early in this project, before we had developed an acceptable Pichinde virus model. To provide rapid feedback to the chemists regarding their compounds, we ran these compounds initially against the Punta Toro virus, another RNA-containing virus of interest to the Department of Defense that was already fully developed. The compounds were also later evaluated against the Pichinde virus once that antiviral model had become established.

B. Materials and Methods

Virus. The An4763 strain of Pichinde virus, obtained from Dr. J. D. Gangemi of the University of South Carolina School of Medicine, was used. The virus was twice plaque-purified in Vero 76 cells and a pool was prepared in those cells. The Adames strain of Punta Toro virus was provided by Dr. Dominique Pifat of the USAMRIID. It was identified as

virus pool #215588 and had been safety-tested by Dr. Pifat before being sent to us. The virus was twice plaque-purified in LLC-MK_2 cells and a pool was prepared in those cells. Both viruses had their identity confirmed by using specific antiserum.

Cells. Cells used in these studies included African green monkey kidney (Vero 76) and Rhesus monkey kidney (LLC-MK_2), obtained initially from the ATCC (Rockville, MD). All were passaged in disposable tissue culture flasks. Both cell lines used minimum essential medium (MEM), obtained from GIBCO (Grand Island, NY), with 5% fetal bovine serum (FBS) and 0.1% $NaHCO_3$.

Test Compounds. Three newly synthesized compounds—1-[5-(3-carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (SRI-7422-52), 1-[2,3-O-(1-carboxy-3-butylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide sodium salt (SRI-7422-80), and 1-[2,3-O-(1-carboxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide sodium salt (SRI-7422-82)—and two samples of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) were evaluated. One ribavirin sample (SRI-7422-56, also designated as SRI-6898-56) was synthesized at SRI International; the other (ICN 1229-38) was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). The ribavirin samples were run as positive controls in the tests. All of the compounds appeared to be soluble in the aqueous medium used in our antiviral tests. Storage of all compounds was in sealed vials, in the presence of desiccant, at room temperature. Once placed in aqueous solution, all were maintained at 4 °C.

In Vitro Antiviral Test Procedures. Two methods were used to determine the antiviral activity of the submitted compounds. These were the inhibition of viral cytopathogenic effect (CPE) and the reduction of viral plaques.

The CPE inhibition studies essentially used the test method that we described previously.[1] In the test with Punta Toro virus, compound was added to an 18-hour monolayer of LLC-MK_2 cells in 96-well microplates. Virus in a concentration of 320 cell culture 50% infectious doses ($CCID_{50}$) per milliliter was added 15 minutes after addition of test compound. The appropriate virus concentrations were determined by preassay in LLC-MK_2 cells. Seven concentrations of each test compound were used: 1000, 320, 100, 32, 10, 3.2, and 1 µg/ml. In later studies these were adjusted to be approximately equimolar, the molar concentrations being 4.1, 1.3, 0.41, 0.13, 0.041, 0.013, and 0.004 mM. The panels were sealed with plastic wrap and incubated at 37 °C until CPE developed (6 days). Three virus-containing cups in each microplate were used for each dosage level, with one cup being used for toxicity controls (cells + sterile virus diluent + compound). Six cups in each panel were used for normal cell controls (cells + sterile virus diluent + drug diluent). Test medium was MEM with 2% FBS, 0.18% $NaHCO_3$, and 50 µg/ml of gentamicin. This medium was also used for virus diluent and for suspending the test compound.

Viral CPE was graded from 0 (normal cells) to 4 (virtually complete destruction of the cell layer). The CPE was read by one individual who was trained in CPE evaluation; then this reading was confirmed by a

second, similarly trained, individual. Reduction in CPE was evaluated by virus rating (VR), as we have described previously[1,2] and by 50% effective dose (ED_{50}).

The VR is a numerical expression of antiviral activity, taking into account percent of CPE inhibition and partial cytotoxicity of the test compound. In our experience, a VR of 1.0 or greater is indicative of definite antiviral activity, a VR of 0.5-0.9 indicates moderate activity, and a VR of < 0.5 suggests slight activity, perhaps resulting from cytotoxicity only.

The ED_{50} was determined by plotting percent CPE inhibition versus test compound concentration on semilogarithmic paper, with the ED_{50} level being that level causing an approximate 50% CPE inhibition.

Also included in the evaluation of each test was an estimated maximum tolerated dose (MTD) of the test compound, this being the highest dosage not causing visually discernible cytotoxic effects in concurrently run toxicity controls. Cytotoxicity was determined by microscopic examination of compound-induced cytopathic effects in treated cultures compared with those in control cells run on the same plates.

Apparent positive activity was validated by fixing the drained cells in 10% formalin and staining them with 1% crystal violet, which clearly demonstrated the complete cell monolayer. The stained plate was labeled and photographed.

Experiments studying the inhibition of CPE caused by Pichinde virus were run using the same methodology as was used for the Punta Toro virus except that Vero 76 cells were used and CPE was read after a 12-day incubation. In this series of experiments, the medium was removed on Day 6 and fresh medium containing compound was added.

Plaque reduction experiments were run as we have described previously.[3] Briefly, a monolayer of Vero 76 cells was exposed to 150 plaque-forming units (PFU) of Pichinde virus incubated for 1 hour at 37° C, and then overlaid with MEM containing 2% FBS, 0.18% $NaHCO_3$, 50 μg/ml of gentamicin, and 1% agarose with the appropriate concentration of test compound in MEM. Seven concentrations of test compound as described above for the CPE inhibition test were used. The cells were incubated for 4 days at 37 °C, then stained with neutral red, and the plaques were counted. An ED_{50} was determined for each compound as described above. Two plates were used for each concentration of test compound and normal controls, and four plates were used for virus controls.

C. Results and Discussion

The effects of the five compounds against Punta Toro virus infections in LLC-MK_2 cells are summarized in Tables V-1, V-2, and V-3. The hemisuccinate of ribavirin (SRI-7422-52) and both lots of ribavirin were run in one experiment. About three months later the sodium levulinate ketal (SRI-7422-80) and sodium 4-acetylbutyrate ketal (SRI-7422-82) of ribavirin were run, with the hemisuccinate and SRI ribavirin being included for comparison purposes. Both lots of ribavirin exerted essentially identical antiviral activity, with VRs of 1.3 and ED_{50}s of

3.2-10 μg/ml. The hemisuccinate in the first test was somewhat less active than ribavirin, with a VR of 0.9 and an ED_{50} of 32 μg/ml. The MTD of this compound, however, was higher than that of ribavirin: 32 μg/ml versus 10 μg/ml, suggesting that the compound was somewhat better tolerated by the cells. In the second Punta Toro virus experiment, the hemisuccinate's activity was more pronounced (VR: 1.3, ED_{50}: 22 μg/ml). Ribavirin's efficacy was also slightly increased (VR: 1.4, ED_{50}: 8 μg/ml), suggesting that the viral inoculum used in the second experiment was somewhat less infectious than that used in the initial test. The ketal derivatives of ribavirin were inactive in the experiment, however.

Ribavirin and the three ribavirin derivatives were also evaluated against Pichinde virus using CPE inhibition. These data are summarized in Tables V-4 and V-5. Pichinde virus, presumably due to the many defective interfering viral particles produced when the virus replicates, does not readily induce CPE. However, if the infected cells are held through 12 days after initial exposure to virus, a 1+ CPE develops that can be read microscopically. In this experiment, ribavirin was active, with a VR of 0.5 and an ED_{50} of 6.0 μg/ml (0.025 mM). The hemisuccinate was again slightly less active, with a VR of 0.4 and an ED_{50} of 14.7 μg/ml (0.041 mM). Against Pichinde virus, the sodium levulinate ketal derivative was slightly active, with a VR of 0.2, whereas the sodium 4-acetylbutyrate derivative failed to exert any discernible antiviral effects.

The Pichinde virus antiviral experiments using CPE inhibition were not considered too satisfactory since the CPE was so slight. We therefore repeated this antiviral experiment using viral plaque reduction as the parameter for evaluation. The results are seen in Tables V-6 and V-7. Ribavirin was highly effective, with an ED_{50} of 2.5 μg/ml (0.024 mM); the hemisuccinate was again slightly less active than ribavirin, although the ED_{50} of 5 μg/ml (0.05 mM) was considered to indicate strong antiviral activity. The ketal derivatives were considered inactive against Pichinde virus using this plaque reduction parameter.

These results indicate that the hemisuccinate addition to the ribavirin molecule did not significantly affect ribavirin's antiviral activity, whereas derivatization to the 2',3'-ketals caused marked loss in the compound's antiviral activity. Although these ketals were inactive in our antiviral models, their behavior after conjugation to monoclonal antibodies and digestion with lysosomal hydrolases of the cell remains to be determined.

These data also indicate that both CPE inhibition and plaque reduction parameters yield essentially the same antiviral results, although the low degree of CPE and its slow development, preclude use of that model for extensive antiviral work with Pichinde virus. The plaque-reduction test, although acceptably reproducible and sensitive, requires much greater quantities of test compound for each evaluation.

We are also considering the use of indirect immunofluorescence, as described in Section II, to discern virally infected cells; preliminary

studies completed to date indicate that this parameter may work well with Pichinde virus.

D. Conclusions

Ribavirin and the 5'-hemisuccinate derivative of ribavirin were found to have highly significant *in vitro* antiviral activity against Punto Toro and Pichinde viruses. The ketal derivatives were inactive. The Punta Toro virus studies utilized a CPE inhibition parameter; the Pichinde virus studies were accomplished using CPE inhibition and plaque reduction in separate experiments. Both parameters yielded similar positive and negative antiviral data, but the CPE produced by Pichinde virus, using the methodology for these tests, is slow to develop and not considered sufficiently extensive to use in antiviral experiments.

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Table V-1. Comparison of Punta Toro Virus-Inhibitory Activity of Ribavirin Hemisuccinate and Ribavirin Using Inhibition of Viral Cytopathic Effects in LLC-MK_2D Cells (USU Experiments SD/PTC 1 to 3).

Cmpd. Conc. (μg/ml)	1-[5-(3-Carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (SRI-7422-52) Cytotox.[a] (%)	Avg. CPE[b]	% Inhib.	SRI Ribavirin (SRI-7422-56) Cytotox. (%)	Avg. CPE	% Inhib.	ICN Ribavirin (ICN 1229-38) Cytotox. (%)	Avg. CPE	% Inhib.
1000	50	0	100	50	0	100	50	0	100
320	25	0	100	50	0	100	50	0	100
100	13	0.2	95	25	0	100	25	0	100
32	0	1.7	54	13	0	100	13	0	100
10	0	2.7	27	0	0.5	86	0	0.3	92
3.2	0	3.5	5	0	2.5	32	0	2.5	32
1	0	3.7	0	0	3.1	16	0	3.3	11
0	—	3.7	—	—	3.7	—	—	3.7	—
VR[c]:		0.9			1.3			1.3	
ED_{50}[d] (μg/ml):		32			3.2-10			3.2-10	
MTD[e] (μg/ml):		32			10			10	

[a] Visible cytotoxicity as seen microscopically, expressed as enlarged, flattened cells. The percent indicated was the approximate degree of enlargement and flattening that occurred.

[b] Cytopathic effect induced by the virus and scored microscopically.

[c] Virus rating, a mathematical expression of antiviral effect, taking into account cytotoxicity and degree of CPE inhibition.[1,2]

[d] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

[e] Maximum tolerated dose: that test compound concentration that induced no visible cytotoxic effect.

Table V-2. Comparison of Punta Toro Virus-Inhibitory Effects of SRI Compounds Using Inhibition of Viral Cytopathic Effects in LLC-MK_2D Cells (USU Experiments SD/PTC 4 and 5).

	1-[2,3-O-(1-Carboxy-3-butylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (SRI-7422-80)				1-[2,3-O-(1-Carboxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (SRI-7422-82)			
Molar Conc. (mM)	Conc. (μg/ml)	Cytotox.[a] (%)	Avg. CPE[b]	% Inhib.	Conc. (μg/ml)	Cytotox. (%)	Avg. CPE	% Inhib.
4.1	1500	0	4	0	1600	0	4	0
1.3	475	0	4	0	507	0	4	0
0.41	150	0	4	0	160	0	4	0
0.13	47.5	0	4	0	51	0	4	0
0.041	15	0	4	0	16	0	4	0
0.013	4.8	0	4	0	5.1	0	4	0
0.004	1.5	0	4	0	1.6	0	4	0
0	0	0	4	0	0	0	4	-
VR[c]:			0.0				0.0	
ED_{50}[d] (μg/ml):			>1500				>1600	
ED_{50} (mM)			>4.1				>4.1	
MTD[e] (μg/ml):			>4.1				>4.1	

[a] Visible cytotoxicity as seen microscopically, expressed as enlarged, flattened cells. The percent indicated was the approximate degree of enlargement and flattening that occurred.

[b] Cytopathic effect induced by the virus and scored microscopically.

[c] Virus rating, a mathematical expression of antiviral effect, taking into account cytotoxicity and degree of CPE inhibition.[1,2]

[d] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

[e] Maximum tolerated dose: that test compound concentration that induced no visible cytotoxic effect.

Table V-3. Comparison of Punta Toro Virus-Inhibitory Effects of SRI Compounds Using Inhibition of Viral Cytopathic Effects in LLC-MK_2D Cells (USU Experiments SD/PTC 6 and 7).

	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (SRI-6898-56)				1-[5-(3-Carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (SRI-7422-52)			
Molar Conc. (mM)	Conc. (μg/ml)	Cytotox.[a] (%)	Avg. CPE[b]	% Inhib.	Conc. (μg/ml)	Cytotox. (%)	Avg. CPE	% Inhib.
4.1	1000	50	0	100	1476	50	0	100
1.3	320	25	0	100	467	25	0	100
0.41	100	12	0	100	148	0	0	100
0.13	32	0	0	100	48	0	0	100
0.041	10	0	0.5	88	14.8	0	3.2	20
0.013	3.2	0	3.8	5	4.8	0	4	0
0.004	1.0	0	4	0	1.5	0	4	0
0	0	0	4	-	0	0	4	-
VR[c]:			1.4				1.3	
ED_{50}[d] (μg/ml):			8				22	
ED_{50} (mM)			0.033				0.04-0.13	
MTD[e] (μg/ml):			0.13				0.41	

[a] Visible cytotoxicity as seen microscopically, expressed as enlarged, flattened cells. The percent indicated was the approximate degree of enlargement and flattening that occurred.

[b] Cytopathic effect induced by the virus and scored microscopically.

[c] Virus rating, a mathematical expression of antiviral effect, taking into account cytotoxicity and degree of CPE inhibition.[1,2]

[d] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

[e] Maximum tolerated dose: that test compound concentration that induced no visible cytotoxic effect.

Table V-4. Comparison of Pichinde Virus-Inhibitory Effects of SRI Compounds Using Inhibition of Viral Cytopathic Effects in Vero 76 Cells (USU Experiments SD/PCC 1 and 2).

	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (SRI-6898-56)				1-[5-(3-Carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (SRI-7422-52)			
Molar Conc. (mM)	Conc. (μg/ml)	Cytotox.[a] (%)	Avg. CPE[b]	% Inhib.	Conc. (μg/ml)	Cytotox. (%)	Avg. CPE	% Inhib.
4.1	1000	50	0	100	1476	50	0	100
1.3	320	25	0	100	467	0	0	100
0.41	100	0	0	100	148	0	0	100
0.13	32	0	0	100	47	0	0.3	70
0.041	10	0	0.3	70	14.7	0	0.5	50
0.013	3.2	0	0.7	30	4.7	0	0.9	10
0.004	1.0	0	1.0	0	1.8	0	1.0	0
0	0	-	1.0	-	0	-	1.0	-
VR[c]:			0.5			0.4		
ED_{50}[d] (μg/ml):			6.0			14.7		
ED_{50} (mM)			0.025			0.041		
MTD[e] (μg/ml):			0.41			1.3		

[a] Visible cytotoxicity as seen microscopically, expressed as enlarged, flattened cells. The percent indicated was the approximate degree of enlargement and flattening that occurred.

[b] Cytopathic effect induced by the virus and scored microscopically.

[c] Virus rating, a mathematical expression of antiviral effect, taking into account cytotoxicity and degree of CPE inhibition.[1,2]

[d] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

[e] Maximum tolerated dose: that test compound concentration that induced no visible cytotoxic effect.

Table V-5. Comparison of Pichinde Virus-Inhibitory Effects of SRI Compounds Using Inhibition of Viral Cytopathic Effects in Vero 76 Cells (USU Experiments SD/PCC 3 and 4).

	1-[2,3-O-(1-Carboxy-3-butylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (SRI-7422-80)				1-[2,3-O-(1-Carboxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (SRI-7422-82)			
Molar Conc. (mM)	Conc. (μg/ml)	Cytotox.[a] (%)	Avg. CPE[b]	% Inhib.	Conc. (μg/ml)	Cytotox. (%)	Avg. CPE	% Inhib.
4.1	1500	50	0.7	30	1600	0	1.0	0
1.3	475	25	1.0	0	507	0	1.5	0
0.41	150	0	0.7	30	160	0	0.8	20
0.13	47.5	0	0.5	50	51	0	1.0	0
0.041	15	0	0.7	30	16	0	1.0	0
0.013	4.8	0	1.0	0	5.1	0	1.0	0
0.004	1.5	0	1.0	0	1.6	0	1.0	0
0	0	-	1.0	-	0	-	1.0	-
VR[c]:			0.2				0.0	
ED_{50}[d] (μg/ml):			>1500				>1600	
ED_{50} (mM)			>4.1				>4.1	
MTD[e] (μg/ml):			0.41				>4.1	

[a] Visible cytotoxicity as seen microscopically, expressed as enlarged, flattened cells. The percent indicated was the approximate degree of enlargement and flattening that occurred.

[b] Cytopathic effect induced by the virus and scored microscopically.

[c] Virus rating, a mathematical expression of antiviral effect, taking into account cytotoxicity and degree of CPE inhibition.[1,2]

[d] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

[e] Maximum tolerated dose: that test compound concentration that induced no visible cytotoxic effect.

Table V-6. Comparison of Pichinde Virus-Inhibitory Effects of SRI Compounds Using Plaque Reduction in Vero 76 Cells (USU Experiments 7 and 8).[a]

	1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (SRI-6898-56)			1-[5-(3-Carboxypropionyl)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide (SRI-7422-52)		
Molar Conc. (mM)	Conc. (μg/ml)	Avg. No. Plaques[b]	% Inhib.	Conc. (μg/ml)	Avg. No. Plaques	% Inhib.
4.1	1000	0	100	1476	no sample	-
1.3	320	0	100	467	0	100
0.41	100	0	100	148	0	100
0.13	32	0	100	47	46	71
0.041	10	10	94	14.7	71	55
0.013	3.2	92	41	4.7	92	41
0.004	1.0	116	26	1.48	102	35
0	0	157	-	0	157	-
ED_{50}[c] (μg/ml):		2.5			5.0	
ED_{50} (mM):		0.024			0.05	

[a] A monolayer of Vero cells was exposed to 150 plaque-forming units of Pichinde Strain An4763, incubated for 1.0 hour at 37° C, and then overlaid with MEM containing 2% FBS, 0.18% $NaHCO_3$, 50 μg/ml of gentamicin, and 1% agarose with the appropriate concentration of drug in MEM. The cells were then incubated for four days at 37 °C before staining with neutral red and counting the plaques.

[b] Average of two plates per concentration.

[c] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

Table V-7. Comparison of Pichinde Virus-Inhibitory Effects of SRI Compounds Using Plaque Reduction in Vero 76 Cells (USU Experiments 7 and 8).[a]

	1-[2,3-O-(1-Carboxy-3-butylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (SRI-7422-80)			1-[2,3-O-(1-Carboxy-4-pentylidene)-β-D-ribofuranosyl]-1,2,4-triazole-3-carboxamide Sodium Salt (SRI-7422-82)		
Molar Conc. (mM)	Conc. (μg/ml)	Avg. No. Plaques[b]	% Inhib.	Conc. (μg/ml)	Avg. No. Plaques	% Inhib.
4.1	1500	130	17	1600	160	0
1.3	475	137	13	506	138	12
0.41	150	122	22	160	138	12
0.13	47.5	133	15	51	156	0
0.041	15	126	20	16	140	11
0.013	4.8	134	15	5.1	136	13
0.004	1.5	144	8	1.6	141	10
0	0	157	-	-	157	-
ED_{50}[c] (ug/ml):		>1500			>1600	
ED_{50} (mM):		>4.1			>4.1	

[a] A monolayer of Vero cells was exposed to 150 plaque-forming units of Pichinde Strain An4763, incubated for 1.0 hour at 37°C, and then overlaid with MEM containing 2% FBS, 0.18% $NaHCO_3$, 50 μg/ml of gentamicin, and 1% agarose with the appropriate concentration of drug in MEM. The cells were then incubated for four days at 37°C before staining with neutral red and counting the plaques.

[b] Average of two plates per concentration.

[c] 50% effective dose: that concentration of test compound that caused an approximate 50% CPE inhibition.

VI. Development of an *In Vivo* Model for Pichinde Virus in MHA Hamsters.

A. Introduction

Pichinde virus has been shown to cause a fatal infection in MHA hamsters that can be used as an *in vivo* model for the evaluation of antiviral regimens.[1-3] The infection in MHA hamsters is similar to some of the hemorrhagic diseases, such as Lassa fever, seen in humans. The target organs in the MHA hamster are primariy the spleen and the liver--specifically, the natural killer cells of the spleen, and the Kupffer cells and hepatocytes of the liver.[2] This section describes results of the titration of a large pool of Pichinde virus in these animals as a step toward preparing an animal model for later antiviral studies.

B. Materials and Methods

Virus. The An4763 strain of Pichinde virus, obtained from Dr. J. D. Gangemi of the University of South Carolina School of Medicine, and the CoAn3739 strain of Pichinde virus, obtained from the ATCC, were used. The viruses were twice plaque-purified in Vero 76 cells and a large pool was prepared in these cells as described in Section II.

Animals. Five-week-old female MHA strain hamsters weighing approximately 60 g were used to titer the virus pool. These hamsters, purchased from Charles River Laboratories, were not designated as specific pathogen-free animals, although they appeared to be in good health. They were housed in shoebox-type polycarbonate cages on a

laminar flow cabinet in the Utah State University Laboratory Animal Research Center. They were fed hamster chow and water *ad libitum*.

Mode of Viral Infection. The hamsters were infected subcutaneously with 0.2 ml of varying tenfold dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) of the virus; the virus diluent was Pucks balanced salt solution. Six hamsters were used in each infected group and 12 were used as uninfected controls. The animals were examined daily and deaths were recorded. The titration was concluded on Day 22 post-virus inoculation.

C. Results and Discussion

The pool of An4763 strain of Pichinde virus that was used to infect the MHA hamsters produced 100% mortality in the 10^{-1} through 10^{-4} -fold dilution infected groups. Mortality was 50% in the 10^{-5} infected group, but there were no deaths in the 10^{-6} and 10^{-7} dilution groups. Mean survival times of those that died ranged from 11 to 13 days. The results are summarized in Table VI-1.

The lethal infection of the MHA hamsters with Pichinde virus strain An4763 was associated with a severe hemorrhagic disease in these animals. The hamsters bled from their eyes, nose, and rectum. The eyes swelled shut and appeared crusty. Gross necropsy of the sacrificed moribund animals revealed a serous infiltrate in the abdominal cavity. The livers of the infected animals appeared pale and severely discolored. The spleens were rigid and enlarged and had a reddish-white, splotchy, appearance. The intestines were dark and distended.

D. Conclusions

The An4763 strain of Pichinde virus, when used to infect the MHA strain of hamsters, produced a lethal infection that can be used as a model for the evaluation of antiviral agents *in vivo*. The range of the mean survival times of those animals that died was 11.0 to 13.5 days. The LD_{50} of the virus was 5×10^5.

The An4763 virus appears to be much more virulent in animals than it is in cell culture. We are confident that this antiviral testing system will meet the needs of this project as put forth in our proposal.

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Table VI-1. Titration of Strain An4763 of Pichinde Virus in Female MHA Hamsters.

Virus Dilution	Survivors/ Total	Mean Survival Time (days)
10^{-1}	0/6	10.9
10^{-2}	0/6	11.0
10^{-3}	0/6	11.2
10^{-4}	0/6	11.1
10^{-5}	3/6	13.5
10^{-6}	6/6	>21.0
10^{-7}	6/6	>21.0

LD_{50}[a] = 10^{-5}

[a] Determined by Reed-Muench.[4]

VII. Development of Methodology Overview for the Derivation and Production of Monoclonal Antibodies against Pichinde Virus-Specified Cell Surface Antigens

A. Introduction

The derivation and production of monoclonal antibodies (MAb) against Pichinde virus-coded antigens expressed on the surface of Pichinde virus-infected cells involves a series of steps, many of which must be accomplished prior to initiating subsequent portions of the process. The necessity for several of these steps may not be obvious, so the following is a brief outline of the steps and the rationale for each step. In Figure VII-1 the steps in anti-Pichinde virus MAb production are outlined in the form of a flow chart, which indicates the sequential nature of many of the steps. Comments and/or explanations relating to the steps to be accomplished in the first page of Figure VII-1, which were primarily accomplished during the first year of this project, are presented below.

B. Methodology Overview and Rationale

In Vitro Culture of Pichinde Virus. Cell culture-grown Pichinde virus is required as an inoculum for the production of Pichinde virus-infected target cells to be used in screening for monoclonal antibodies and as the inoculum for the *in vivo* drug-screening procedures. Cell culture-grown Pichinde virus is also the inoculum of choice for the *in vivo* compound screenings. It is easier to establish the purity of a cell

culture-derived pool of pure Pichinde virus, than that of an organ homogenate pool that might contain other viruses or bacteria.

Plaquing of Pichinde Virus. All of our studies will utilize Pichinde virus that has been plaque-purified twice. This assures that we are looking at the effects of Pichinde virus and not some opportunistic contaminant. In addition, since Pichinde virus is so difficult to assay by end-point cytopathic effect, many of our quantitative virus assays will utilize the plaque assay procedure and an indirect immunofluorescence assay procedure.

Production of a Cell Culture Pool of Pichinde Virus. The twice-plaque-purified Pichinde virus (strain An4763) will be used to produce a pool of Pichinde virus. This will be done by inoculating Vero 76 cells with virus at an MOI of 1 PFU/cell. The infections will be stopped by freezing 3 days post-virus exposure. After three cycles of freeze/thaw, the cell culture fluids will be centrifuged at 1900 RPM for 10 minutes. The supernates, free of large cell debris, will be pooled and dispensed in aliquots and stored at $-80^{\circ}C$. This virus preparation will be titered by plaque formation, by indirect immunofluorescence assay, and in MHA hamsters. If the titers are sufficiently high, this virus pool will provide the inocula for *in vitro* and *in vivo* antiviral experiments.

Growth and Purification of a Large Quantity of Pichinde Virus. From the twice-plaque-purified Pichinde virus stock a large pool of Pichinde virus will be produced in roller cultures and stationary cultures of Vero cells. Once purified by differential and isopycnic centrifugation, this

Pichinde virus will serve as the antigen for determining the anti-Pichinde virus MAb peptide and epitope specificity.

Preparation of Immunogens for Spleen Donor Mice. Suckling (6-day-old) RBF/DnJ and BALB/c mice are to be inoculated intracerebrally with 10 µl of twice-plaque-purified An4763 Pichinde virus. From each pool of suckling mouse brains, a 10% homogenate in PBS will to be prepared. Mouse brain pools of Pichinde virus are used as immunogens in the spleen-donor mice. We are using two strains of mice for monoclonal antibody production; our primary effort will be with the RBF/DnJ strain of mice, which are the spleen-donor partners to the FOX-NY myeloma cell line that we are utilizing. Our backup strain of mouse is the BALB/c, which is the spleen donor for fusions utilizing the SP2/0-Ag14 myeloma cell line. Spleen donor mice could have been immunized with cell culture-grown Pichinde virus, but then we could expect an immune response to the Vero cell antigens as well as to Pichinde virus antigens. The presence of antibodies to Vero cell antigens would add confusion to the monoclonal antibody screening process, which utilizes virus-infected Vero cells as a target. Thus the choice of a suckling mouse brain homogenate as the immunogen. Also, for the purpose of eliciting as specific a response as possible, the spleen donor mice were immunized with homogenates derived from syngeneic mice, which explains the preparation of Pichinde virus pools in both the RBF/DnJ and the BALB/c strains of mice.

Hyperimmunization of Spleen Donor Mice. The RBF/DnJ suckling mouse and BALB/c suckling mouse brain homogenates will be used to immunize adult RBF/DnJ and BALB/c mice, respectively, with Pichinde virus. The immunogen will be mixed with complete Freund's adjuvant for primary immunizations

and incomplete Freund's adjuvant for booster immunizations, both injected intramuscularly.

Production of Anti-Pichinde Virus Antisera. These antisera are used as positive controls in the development of anti-Pichinde virus antibody detection procedures. Antisera will be produced in mice hyperimmunized with Pichinde virus-infected suckling mouse brain homogenates. Attempts will also be made to produce hyperimmune antisera in BALB/c mouse ascites fluids.

Development of Anti-Pichinde Virus Antibody Detection Procedures. The specific antibody detection procedures must detect antibody specific for Pichinde virus antigens expressed on the surfaces of Pichinde virus-infected cells. Conventional ELISA or radioimmunoassay procedures using cell lysates or purified Pichinde virus as the solid phase are not suitable as screening procedures because they are not specific for Pichinde virus antigens expressed on the cell surface. Several screening procedures as described in the following sections will be investigated.

Fluorescent Antibody Assay for Pichinde Virus or Antibody to Pichinde Virus. Acetone-fixed, Pichinde virus-infected Vero cells yield excellent fluorescent antibody (FA) staining using either direct or indirect FA procedures (see Section X of this report). Although acetone is the fixative of choice for assaying for Pichinde virus via FA, it is not suitable for FA procedures directed at detecting antibody against cell-surface antigens. Several nonpermeabilizing fixatives will be studied to determine which would be most efficacious for these studies.

In our procedure, 96-well microtiter plates containing alternating rows of Pichinde virus-infected and noninfected Vero cells are to be used to detect antibody to Pichinde virus cell-surface antigens and to detect and avoid selection of antibodies that react with noninfected Vero cells.

ELISA, Dot-Blot Assay for Screening for Antibody to Pichinde Virus. The ELISA procedure using Pichinde virus-infected cells as the target in a Bio-Rad 96-well BIO-DOT apparatus will be investigated. As a target we will try both glutaraldehyde-fixed Vero cells and nonfixed Vero cells. The indirect ELISA using a labeled anti-mouse reagent will be used.

Procedures for Screening Hybridomas for Antibody to Pichinde Virus. The most critical step in the derivation of monoclonal antibodies against Pichinde virus is the specific antibody selection procedure used in the screening of the hybridoma supernates. About ten days after the fusion of the myeloma cells with the splenocytes from the hyperimmunized mice, those wells showing cell growth will be screened for antibody specific to Pichinde virus antigens expressed on the surface of Pichinde virus-infected cells. The screening procedure must be specific, sensitive, and relatively simple since thousands of samples will be assayed and false positives will add much unnecessary work at the cloning stage of hybridoma derivation.

Prepararation of Target Cell Plates for Hybridoma Screening. Glutaraldehyde-fixed target cells in 96-well plates, containing alternating rows of Pichinde virus-infected and noninfected Vero cells, are prepared ahead of time. This reduces the problems of coordinating

the preparation of target cells with the care and screening of the fusion cultures.

Culturing of the FOX-NY Cell Line. The FOX-NY cell line was isolated as a spontaneous mutant of the NS-1 myeloma line. The advantages of using the FOX-NY cell line and an abbreviated outline of the hybridization procedures that we are using is described in the original reference to the technique.[1] The utilization of the FOX-NY cell line in fusions with mouse spleen cells containing Robertsonian 8.12 translocation chromosomes has come to be termed Taggart Hybridoma Technology.

When the FOX-NY cell line is brought into a laboratory, its growth characteristics should be determined in the media that will be used for maintenance of the cell line. A population doubling time of 18 to 24 hours should be achieved when the cells are in log-phase growth. Healthy, vigorously growing cells are required for successful fusions.

Establishment of Fusion Techniques. The techniques involved in fusing a myeloma cell to a spleen cell vary widely among laboratories and in the literature. The process of hybridoma derivation is quite labor-intensive and protracted; there are many critical steps at which the hybrids may be lost as a result of either contamination or untimely feedings or manipulations. There are many alternatives in selection of both materials and methods: for example, the choice of myeloma cell line, whether or not to use macrophages in the fusion wells, whether or not to use feeder cells at the cloning steps, the type of fusogen to use.

Consequently, the fusion technique needs to be established, practiced, and proven to yield hybridomas.

Fusion to Generate Hybridomas. In the procedure that we will be using, the FOX-NY cells are fused via polyethylene glycol to spleen cells of hyperimmunized RFB/DnJ strain mice. These mice are characterized by a mutation resulting in a Robertsonian 8.12 translocation, which places portions of chromosomes 8 and 12 on a single 8.12 translocation chromosome. The significance of this is that the gene coding for adenosine phosphoribosyltransferase (APRT) is on chromosome 8 and the heavy-chain immunoglobulin locus is on chromosome 12. Thus the enzyme marker sensitive to the selection procedure is genetically linked to the ability to produce immunoglobulin, with the overall outcome being the generation of stable antibody-producing hybridomas. The fused cells are distributed to 24-well plates at 1×10^6 cells/well.

Selection of Anti-Pichinde Virus Producers. This is the most critical step in the entire monoclonal antibody production scheme. Each well of the plates contains hundreds or even thousands of hybridomas growing in competition with each other. To isolate the desired hybridomas, those wells that contain desirable antibody are cloned into other plates, resulting in an expansion that yields 96 wells from a single selected well. Positive wells from the 96-well first cloning plate are cloned into another 96-well plate, thus resulting in further expansion of the number of wells to be screened. There is no way to keep any more than a small fraction of the resulting hybridomas; the choice as to which to maintain must be made within a few days or the desired hybridomas may be lost as a result of overgrowth by more competitive

hybridomas in the culture. Freezing of nontested hybridomas for later evaluation may severely affect their subsequent growth.

C. Literature Cited

1. Taggart, R. T. and Samloff, I. M. 1983. Stable antibody-producing murine hybridomas. Science, 219:1228-1230.

Figure VII-1. Anti-Pichinde Virus (PCV) Monoclonal Antibody Production

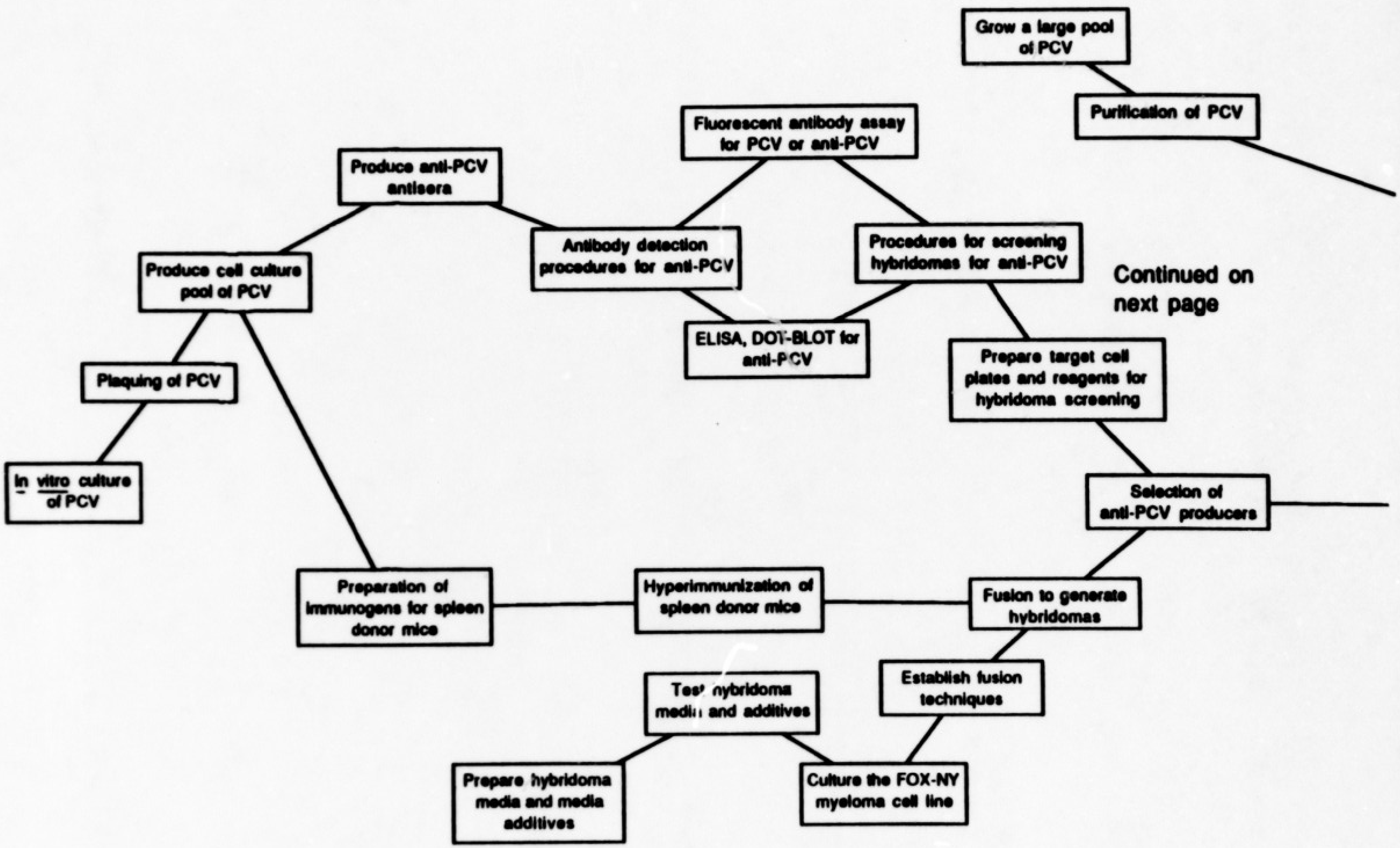

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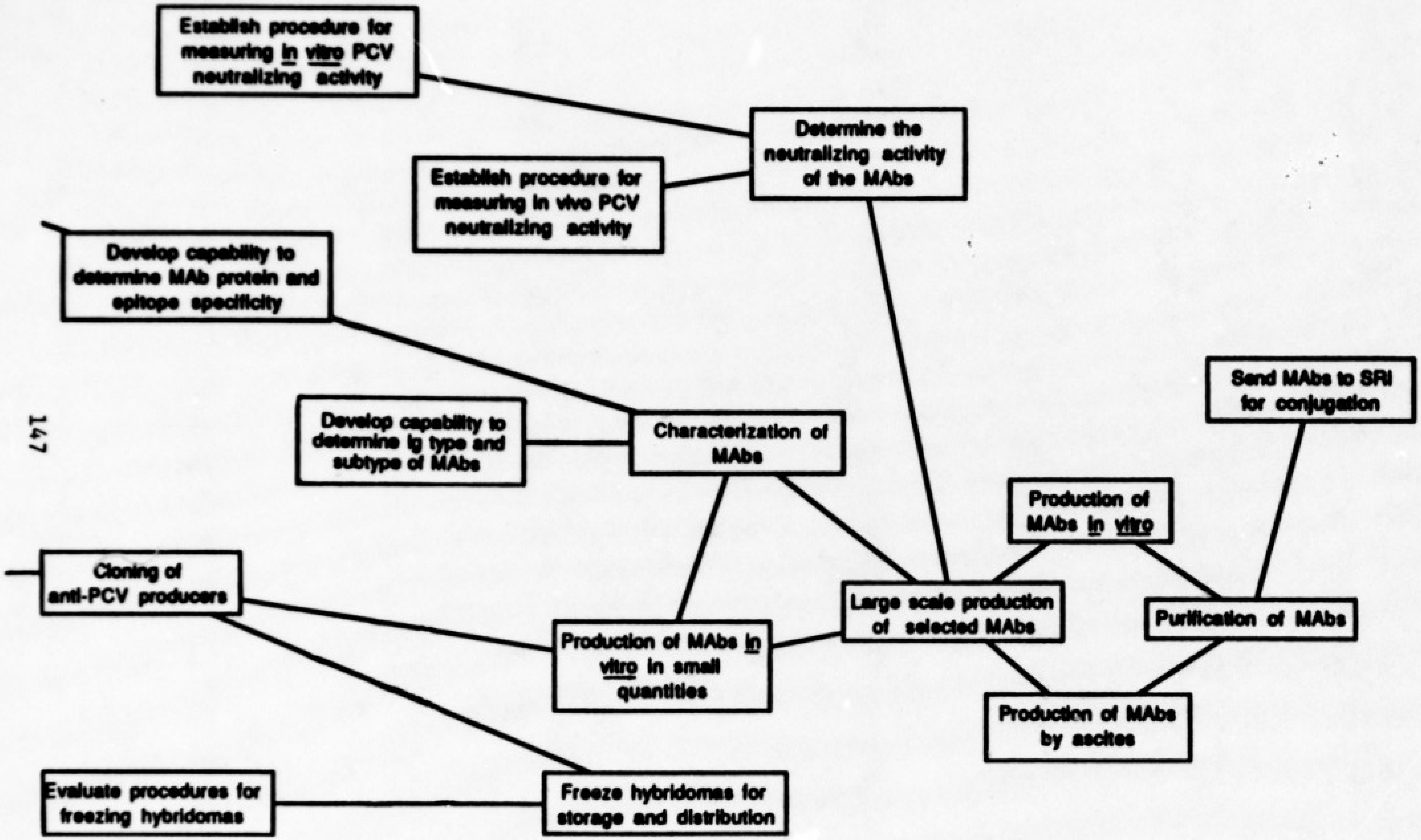
Figure VII-1, continued



VIII. Preparation of Immunogens and Hyperimmunization of Spleen Donor Mice

A. Introduction

For fusions utilizing the FOX-NY myeloma cell line, the RBF/DnJ mouse is the appropriate spleen donor. For the Sp2/0-Ag14 myeloma cell line, the BALB/c mouse is a more suitable spleen donor. Spleen donor mice should not be immunized with cell culture-grown Pichinde virus, as this would quite likely result in an immune response directed at Vero cell antigens as well as the desired Pichinde virus antigens. This would be undesirable since infected Vero cells are the target for the monoclonal antibody screening procedures. Therefore, an immunogen was prepared from the brains of Pichinde virus-infected, syngeneic suckling mice. The suckling mouse brain pools were used to immunize spleen donor mice for the production of anti-Pichinde virus monoclonal antibodies.

B. Materials and Methods

Cells. Cells used in this study were the Vero 76 line of African green monkey kidney cells obtained from the ATCC. All cells were passaged in polystyrene disposable cell culture flasks. Medium for the cells was MEM (GIBCO) containing 9% FBS.

Virus. The An4763 strain of Pichinde virus described in Section II was used in these studies.

Virus Assays. Pichinde virus was assayed by either the plaque assay or indirect immunofluorescence assay procedures as described in Section II of this Report.

Serum Antibody Assay. Serum antibody titers toward Pichinde virus were measured by the indirect immunofluorescence assay procedure using varying dilutions of the mouse sera as the primary antisera. The titer was expressed as the highest dilution that still resulted in specific staining of Pichinde virus-infected cells.

Animals. RBF/DnJ mice obtained from Jackson Laboratories (Bar Harbor, ME) and BALB/c mice obtained from Simonsen Laboratories (Gilroy, CA) were used in these studies.

Preparation of Pichinde Virus Immunogen. Suckling (six-day-old) RBF/DnJ and BALB/c strains of mice were inoculated intracerebrally with 10 µl (5×10^4 plaque-forming units) of twice-plaque-purified An4763 Pichinde virus. The surviving mice were sacrificed on Day 9. Infected suckling mouse brain pools were prepared as 10% homogenates in PBS, pH 7.0.

Immunization of Spleen Donor Mice. The RBF/DnJ suckling mouse brain homogenate and BALB/c suckling mouse brain homogenate were used to immunize eight-week-old RBF/DnJ and BALB/c mice, respectively, with Pichinde virus. The immunogen was mixed 50/50 with complete Freund's adjuvant to yield a stable emulsion, 0.25 ml of which was then injected intramuscularly into each hind flank. The mice were boosted with a suspension of the appropriate syngeneic suckling mouse brain homogenate

in Freund's incomplete adjuvant at six weeks and again at 20 weeks after the primary immunizations.

C. Results and Discussion

Preparation of Immunogen in Suckling Mice. The suckling RBF/DnJ mice were severely affected by the Pichinde virus, showing extreme trembling and a rigid paralysis of the hind limbs at 7 to 8 days post-inoculation. Seven out of nine mice died between Days 7 and 9. The surviving mice were sacrificed on Day 9. A parallel group of suckling BALB/c mice subjected to the same protocol showed much milder symptoms, although one out of ten died. The RBF/DnJ brain homogenate was assayed for Pichinde virus and found to have a titer of 3×10^3 PFU/ml; the BALB/c brain homogenate had a Pichinde virus titer equal to or less than 10 PFU/ml.

Serum Antibody Titers toward Pichinde Virus. The anti-Pichinde virus serum antibody titer in pooled sera from two of the RBF/DnJ mice 21 weeks after the primary immunization was 1:6,400, whereas those of two separate BALB/c mice were 1:100 and 1:200 (Table VIII-1).

D. Conclusions

The RBF/DnJ mice hyperimmunized to serve as spleen donors for anti-Pichinde virus monoclonal antibody production have a very high antibody titer toward Pichinde virus and should be satisfactory spleen donors. The

BALB/c mice similarly immunized had much lower serum antibody titers. This difference is probably a result of the higher Pichinde virus concentration in the immunogen used to immunize the RBF/DnJ mice.

Based on the severity of their response, RBF/DnJ suckling mice inoculated by intracerebral injection with Pichinde virus might serve as a useful model for arenavirus encephalitis.

Table VIII-1. Antibody Titers[a] toward Pichinde Virus in Sera from Hyperimmunized Mice 21 Weeks after Primary Immunization with Pichinde Virus.

Mouse Strain	Serum Dilution[b]	Nonspecific Reaction[c]	Specific Reaction[d]
RBF/DnJ (pool of 2)	1:100	-	+++
	1:400	-	++
	1:1,600	-	++
	1:6,400	-	+
	1:25,600	-	±
	1:102,400	-	-
BALB/c (mouse #1)	1:50	-	++
	1:100	-	+
	1:200	-	+
	1:400	-	±
	1:800	-	-
BALB/c (mouse #2)	1:50	-	+
	1:100	-	+
	1:200	-	±
	1:400	-	±
	1:800	-	-

[a] Antibody measured by indirect immunofluorescence assay using indicated dilutions of test sera as primary antibody and fluorescein-labeled goat anti-mouse immunoglobulin as second antibody, with Pichinde virus-infected Vero 76 cells as targets.

[b] Test sera diluted in phosphate-buffered saline, pH 7.0, were incubated on the target cells for 1 hour at 37°C.

[c] Nonspecific reaction scored from - (no nonspecific staining visible) to +++ (severe nonspecific staining overwhelming any specific staining).

[d] Specific reaction scored from - (no visible specific staining) to +++ (the most intense specific staining resulting from any hyperimmune sera tested, easily visible).

IX. Production of Anti-Pichinde Virus Antisera

A. Introduction

Antisera toward Pichinde virus are required for the development of immunoassays for Pichinde virus and to serve as positive controls in the development of anti-Pichinde virus monoclonal antibody screening procedures. Therefore, both hyperimmune mouse sera and hyperimmune ascites fluids were produced in mice hyperimmunized by intramuscular injections with Pichinde virus.

B. Materials and Methods

Cells. Cells used in this study were the Vero 76 line of African green monkey kidney cells obtained from the ATCC, and the murine myeloma cell line SP2/0-Ag14 obtained from Dr. Mark C. Osterling of USAMRIID.

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi) was used in these studies. The virus was described in Section II.

Virus Assays. Pichinde virus was assayed by either the plaque assay or indirect immunofluorescence assay procedures as described in Section II of this report.

Antibody Assays. Antibody titers toward Pichinde virus were measured by the indirect immunofluorescence assay procedure (as described

in Section II of this report) using varying dilutions of the test sera as the primary antisera. The titer was expressed as the highest dilution that resulted in specific staining of Pichinde virus-infected cells. The second antibody was goat anti-mouse IgG fluorescein conjugate (Boehringer Mannheim Biochemicals).

Animals. Female C57Bl/6 and BALB/c mice six- to nine-weeks old were obtained from Simonsen Laboratories. The animals were housed in shoebox-style polycarbonate cages measuring 10" x 17" x 6" and containing Sanicell bedding. Wayne mouse chow and water were provided *ad libitum*.

Pichinde Virus Immunogen. The immunogens used in these studies were strain An4763 Pichinde virus-infected RBF/DnJ and BALB/c suckling mouse brain homogenates (10% in PBS, pH 7.0), prepared as described in Section VIII of this report, and similar spleen homogenates prepared from the same Pichinde virus-infected RBF/DnJ suckling mice.

Immunization of Antibody Donor Mice. Eighteen C57Bl/6 mice were immunized with Pichinde virus-infected RBF/DnJ suckling mouse brain homogenate. The immunogen was mixed 50/50 with complete Freund's adjuvant, and 0.25 ml was administered by intramuscular injection into each hind flank. The C57Bl/6 mice were boosted similarly with a suspension of the Pichinde virus-infected RBF/DnJ suckling mouse spleen homogenate in Freund's incomplete adjuvant at six weeks after the primary immunizations. Three BALB/c mice were immunized with Pichinde virus as above except that the primary and booster immunogens were prepared from infected BALB/c suckling mouse brain homogenates.

Serum Collection. Two weeks after the booster immunization, the C57Bl/6 mice were anesthetized with ether and exsanguinated by severing the subclavian artery. The serum (approximately 0.4 ml/mouse) was harvested by centrifugation and stored in small aliquots at -80° C.

Ascites Production. Six weeks after receiving the booster immunization, the BALB/c mice were primed by intraperitoneal injection of 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) obtained from Sigma Chemical Co. (St. Louis, MO). Nine days after pristane priming, the mice were inoculated by intraperitoneal injection with 1 x 10^6 SP2/0-Ag14 cells per mouse. The SP2/0-Ag14 cells were suspended at 1 x 10^6 cells per ml in MEM without FBS. The cell suspension consisted of over 95% viable cells as determined by trypan blue staining. Three to four weeks after inoculation of the myeloma cells, ascites fluids were collected by syringe (18-gauge needle) from the swollen abdomens of the donor mice.

C. Results and Discussion

Preparation of Antibody in Adult Mice. None of the immunized adult mice exhibited any ill effects associated with the injections of Pichinde virus-infected BALB/c suckling mouse brain homogenate.

Serum Antibody Titers toward Pichinde Virus. The anti-Pichinde virus serum antibody titer in pooled sera from the C57Bl/6 mice 8 weeks after the primary immunization was 1:640 (Table IX-1).

Ascites Production and Ascites Antibody Titers toward Pichinde Virus. Three weeks after receiving the myeloma cells, one of the mice died. This mouse had developed solid, lumpy tumors in the abdomen, but no ascites fluids. At four weeks after receiving the myeloma cells, the remaining two mice were beginning to show swelling of the abdomen, but it was not nearly as large as a normal pregnant mouse nearing term. At that time a second mouse died; 3 ml of ascites fluids was collected from this dead mouse (referred to as mouse #2), and 1.3 ml of ascites fluids was collected from the surviving mouse. The following day the surviving mouse had ruffled fur and began to exhibit slight tremors; an additional 1.3 ml of ascites fluids was collected. One week later this mouse was much more swollen and 3.0 ml of ascites fluids was collected. This last surviving mouse (referred to as mouse #3) died 3 days later with no further ascites being harvested. The anti-Pichinde virus antibody titer in the ascites collected from the dead mouse was 1:100 and that of the pooled ascites collected from the living mouse was 1:200 (Table IX-1).

D. Conclusions

This study was successful in producing anti-Pichinde virus reagents. Both the ascites fluids and the hyperimmune mouse sera served well when used for immunoassays for Pichinde virus and as positive controls in the development of anti-Pichinde virus monoclonal antibody screening procedures. However, both the hyperimmune mouse sera and the ascites fluids had low anti-Pichinde virus titers (1:640 and 1:200, respectively) when compared with the titer of 1:6,400 of the sera collected from the hyperimmunized RBF/DnJ spleen donor mice (described in Section VIII). The RBF/DnJ spleen donor mice were immunized and boosted twice with the

RBF/DnJ suckling mouse brain homogenate, which was the immunogen with the highest Pichinde virus concentration of all of our immunogens. Low titers in the BALB/c homogenates were used and these had very low Pichinde virus titers. If the ascites fluids antibody titers were higher, then ascites production would have an advantage over hyperimmune mouse serum production in that the ascites yields larger volumes of antisera per mouse. The titers in the C57Bl/6 would probably have been higher if the booster immunogen had contained higher concentrations of Pichinde virus antigens (RBF/DnJ spleen homogenate was used) and if more injections with booster had been administered.

Table IX-1. Antibody Titers[a] toward Pichinde Virus in Hyperimmunized Antibody Donor Mice.

Mouse Strain, Fluid Tested	Dilution[b]	Nonspecific Reaction[c]	Specific Reaction[d]
BALB/c	1:50	+/-	+
(ascites fluids,	1:100	-	+
mouse #2)	1:200	-	+/-
	1:400	-	+/-
	1:800	-	-
	1:1,600	-	-
BALB/c	1:50	+/-	++
(pooled ascites	1:100	-	+
fluids,	1:200	-	+
mouse #3)	1:400	-	+/-
	1:800	-	-
	1:1,600	-	-
C57Bl/6	1:10	+	+++
(pooled serum)	1:40	+/-	++++
	1:160	-	+++
	1:640	-	+
	1:2,560	-	-

[a] Antibody measured by indirect immunofluorescence assay using indicated dilutions of test sera as primary antibody and fluorescein-labeled goat anti-mouse immunoglobulin as second antibody, with Pichinde virus-infected Vero cells as targets.

[b] Ascites fluids and sera diluted in phosphate-buffered saline, pH 7.0, were incubated on the target cells for 1 hour at 37 °C.

[c] Nonspecific reaction scored from - (no nonspecific staining visible) to +++ (severe nonspecific staining overwhelming any specific staining).

[d] Specific reaction scored from - (no visible specific staining) to ++++ (the most intense specific staining resulting from any hyperimmune sera tested); ++ specific reaction is easily visible and suitable as primary antibody for immunofluorescence assay.

X. Development of Fluorescent Antibody Assay for Pichinde Virus or for Antibody to Pichinde Virus

A. Introduction

A simple rapid procedure for the quantitative assay of Pichinde virus was needed for the assay of Pichinde virus in cell culture fluids and animal tissue homogenates. Cytopathogenic effect (CPE), in the case of Pichinde virus, was slow to develop; the Pichinde virus assay based on CPE required 10 to 14 days before a virus titer was readable. While the Pichinde virus plaque assay could be read in 4 to 5 days, an even more rapid procedure for determining infectious Pichinde virus concentrations was desirable. An indirect immunofluorescence assay that could be used as an assay for virus and as a screening procedure for monoclonal antibodies that react with Pichinde virus antigens was developed.

B. Materials and Methods

Cells. The Vero 76 line of African green monkey kidney cells obtained form the ATCC was used for the culture of Pichinde virus and as the target cells for all Pichinde virus assays. All cells were passaged in polystyrene disposable cell culture flasks using MEM (GIBCO, Grand Island, NY) containing 9% FBS (HyClone Labs, Logan, UT). Cells were seeded in several different configurations for these experiments. The Vero cells were seeded at 6 x 10^4 cells/well when seeded in 96-well cell culture plates, 2 x 10^5 cells/well when seeded in 24-well cell culture plates, and at 2 x 10^5 cells/15-mm coverslip when coverslip cultures were required.

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC), and the CoAn3739 strain of Pichinde virus (obtained from ATCC) were used in these studies.

Virus Assays. Pichinde virus was assayed by either the plaque assay described in Section II of this report or by the immunofluorescence assay described in this section.

Antibody Assays. Antibody titers toward Pichinde virus were measured by the indirect immunofluorescence assay procedure, using varying dilutions of the test sera as the primary antisera. The titer was expressed as the highest dilution that still resulted in specific staining of Pichinde virus infected cells. The various second antibody reagents listed in Table X-1 were used. Optimum results were obtained using goat anti-mouse IgG fluorescein conjugate (Boehringer Mannheim Biochemicals).

Antisera. In this study, several anti-Pichinde virus antisera were used; the majority of work utilized hyperimmune anti-Pichinde virus sera (titer of 1:640 by indirect immunofluorescence assay) from adult C57Bl/6 mice prepared as described in Section IX of this report. For the direct immunofluorescence assay, we used fluorescein-conjugated guinea pig antibody towards Pichinde virus (Conj #234; 4/84) obtained from Dr. Meir Kende of USAMRIID (Frederick, MD).

Production of Pichinde Virus-Infected Cells. Growth media was aspirated from cell sheet and viral inoculum was added, virus was allowed to absorb for one hour. Maintenance media, consisting of MEM with 2% FBS, was added, and the culture was incubated at 37°C for 20 to 24 hours and then fixed. The virus inocula volumes were: 100 µl/well in 96-well cell culture plates, 250 µl/well in 24-well cell culture plates, and 20 µl/15-mm coverslip. Pichinde virus was diluted in MEM with 2% FBS.

Fixation. At the end of the 20- to 24-hour incubation, the media was aspirated and the infected cell cultures were air-dried for 30 minutes, then fixed. Cells on glass coverslips were fixed for five minutes in cold acetone (-15°C) and then air-dried prior to staining. Cells in 96- or 24-well cell culture plates were fixed in 80% acetone by adding 50 µl of cold (4°C) phosphate buffered saline/well followed by 200 µl of cold (-15°C) acetone/well. The fixation was done on the bench at room temperature with cold reagents. Cells were either stained immediately or stored for up to two months at -15°C.

Immunofluorescent Staining of Pichinde Virus Infected Cells. Volumes of reagents differed, depending on cell culture configuration: 50 µl/well for 96-well cell culture plates, 200 µl/well in 24-well cell culture plates, and 200 µl/15-mm coverslip. For the direct immunofluorescence assay, fluorescein-labeled antibody toward Pichinde virus was added and incubated at 37°C for one hour. The conjugate was poured off, the cell sheet was rinsed very gently in deionized water, then a drop of either glycerol or elvanol mounting media was added, and the sheet was viewed with a fluorescence microscope. For the indirect immunofluorescence assay, the fixed cells were incubated with mouse anti-

Pichinde virus, diluted in PBS, for one hour at 37°C. The first antibody was removed and the cells were very gently rinsed three times in PBS. Then fluorescein-labeled anti-mouse gamma globulin diluted in PBS was added to the moist cell sheet. After incubation at 37°C for 1 hour, the conjugate was removed, the cell sheet rinsed once in deionized water, and a drop of either glycerol or elvanol mounting media was added. When the cells were in 24-well cell culture plates, a 15-mm coverslip was placed over the drop of elvanol. When coverslip cultures were used, the coverslips were mounted cell-side down on a drop of elvanol after the conjugate was removed.

Viewing. Stained cells in cell culture plates were viewed using a 16X objective and 10X eyepieces with an epifluorescence microscope; coverslip cultures could be viewed with higher powered objectives.

C. Results and Discussion

The inoculation, incubation, and fixation conditions were worked out using the direct immunofluorescence assay with fluorescein-conjugated guinea pig antibody toward Pichinde virus. Incubation periods longer than 28 hours resulted in secondarily infected cells that increased the difficulty of reading the tests; incubation periods of less than 18 hours resulted in only sparse antigen development, again increasing the difficulty of reading the test.

The indirect immunofluorescence assay was then developed using hyperimmune mouse sera (HMS). Using this HMS, several commercially

obtained fluorescein-conjugated anti-mouse immunoglobulin conjugates were evaluated for specificity and potency. The results of these second-antibody screenings are shown in Table X-1. As a result of these observations, we used the goat anti-mouse gamma globulin fluorescein-labeled reagent from Boehringer Mannheim Biochemicals. The specificity of the indirect immunofluorescence assay was confirmed by comparing immunofluorescent cell counts to those obtained using fluorescein-conjugated guinea pig anti-Pichinde virus antibody in the direct immunofluorescence assay. The counts were the same with either assay procedure.

A drop of elvanol mounting medium placed in the well after the second antibody was removed preserved the intensity and clarity of the stained specimen for later viewing or for photography. However, the addition of elvanol was not necessary for viewing fluorescent cells, especially if the samples were examined within a few hours. The cells in wells with elvanol were clearer and sharper than those in wells without elvanol. Stained cell cultures mounted in elvanol and stored at $-15^{\circ}C$ in the dark for two months retained at least 75% of their clarity and intensity.

The indirect immunofluorescence assay was used to determine mouse serum and ascites fluid antibody titers toward Pichinde virus. Varying dilutions of ascites fluids or sera, in PBS (pH 7.0), were substituted for the primary antibody in the indirect immunofluorescence assay. The anti-Pichinde virus antibody titer was the greatest dilution that still gave easily recognized immunofluorescent staining of Pichinde virus-infected Vero cells. An example is seen in Table IX-1 of this report.

Mouse and hamster organ homogenates as well as cell culture fluids were assayed for infectious Pichinde virus by the indirect immunofluorescence assay. In Table X-2 the Pichinde virus titers of three cell culture pools determined by the indirect immunofluorescence assay are compared to the titers determined by plaque assay, cytopathic effect, and infectivity assay in MHA hamsters. All of these assays are described in previous sections of this report.

D. Conclusions

The immunofluorescence procedures described in this report can be used to reliably assay infectious Pichinde virus in a wide range of sample matrices more economically and in a shorter time than either the cytopathic effect or plaque assay procedures. Indirect immunofluorescence can be used in either qualitative or quantitative assays for antibody to Pichinde virus. Acetone-fixed Pichinde virus-infected Vero cells may be prepared in advance and stored at $-15^{\circ}C$ prior to serving as targets for fluorescent antibody staining.

Table X-1. Comparisons[a] of Fluorescein-Conjugated Antisera toward Mouse Immunoglobulin.

Antibody[b]	Dilution[c]	Nonspecific Reaction[d]	Specific Reaction[e]
Ab 1	1:100	±	+++
Ab 1	1:200	-	++
Ab 1	1:400	-	+
Ab 1	1:800	-	±
Ab 2	1:100	++	+
Ab 2	1:200	+	-
Ab 2	1:400	+	-
Ab 2	1:800	-	-
Ab 3	1:100	-	±
Ab 3	1:200	-	-
Ab 3	1:400	-	-
Ab 3	1:800	-	-
Ab 4	1:100	-	±
Ab 4	1:200	-	-
Ab 4	1:400	-	-
Ab 4	1:800	-	-
Ab 5	1:100	±	++
Ab 5	1:200	-	+
Ab 5	1:400	-	±
Ab 5	1:800	-	-
Ab 6	1:50	+	+++
Ab 6	1:100	±	+++
Ab 6	1:200	-	++++
Ab 6	1:400	-	++++
Ab 6	1:800	-	+++
Ab 6	1:1600	-	+++
Ab 6	1:3200	-	++

[a] Antisera compared in an indirect immunofluorescence assay using 1:100 dilution of hyperimmune mouse sera and the indicated dilutions of fluorescein-labeled anti-mouse immunoglobulin as second antibody with Pichinde virus-infected Vero cells as targets.

[b] Ab 1: Goat anti-mouse gamma globulin, fluorescein-labeled, Lot No. 8F, Antibodies Incorporated, Davis, CA.

Ab 2: Rabbit anti-mouse, Cat. No. CL 6002-F, Cedarline, Hornby, Ontario, Canada.

Ab 3: Fluorobody goat antibody to mouse IgG (H+L), Cat. No. 8608-13, Lot No. AC024, Bionetics, Kensington, MD.

Ab 4: Goat antibody to mouse IgG (H+L) affinity purified, Cat. No. 401214, Lot No. 202306, CalBiochem, La Jolla, CA.

Ab 5: FITC anti-mouse IgG (H+L), Cat. No. 51061A, Lot No. MDF006, HyClone, Logan, UT.

Ab 6: Goat anti-mouse IgG fluorescein conjugate, Cat. No. 605 240, Lot No. 8F, Boehringer Mannheim Biochemicals, Indianapolis, IN.

[c] Diluted in phosphate-buffered saline, pH 7.0, and incubated on the target cells for 1 hour at 37°C.

[d] Nonspecific reaction scored from - (no nonspecific staining visible) to +++ (severe nonspecific staining overwhelming any specific staining).

[e] Specific reaction scored from - (no specific staining) to ++++ (the most intense specific staining resulting from any reagents tested); ++ is easily visible and suitable for immunofluorescence assay.

Table X-2. Pichinde Virus Titers Determined by Immunofluorescence, Plaque Assay, Cytopathic Effect, and *In Vivo* Assay in Hamsters.

Virus Pool	Fluorescence IFCFU[a]	Plaque PFU[b]	CPE $TCID_{50}$[c]	Hamster LD_{50}[d]
PCV CoAn3739 (9/29/86)	2.6×10^8	2×10^7	2×10^7	
PCV An4763 (6/3/86)	1.6×10^6	4×10^5		5×10^5

[a] Immunofluorescent cell forming units/ml.

[b] Plaque forming units/ml.

[c] Tissue culture infectious doses/ml, determined in Vero 76 cells.

[d] Number of lethal dose units/ml, determined in MHA hamsters.

XI. Development of Detection Procedures Specific for Antibodies toward Cell Surface-Associated Pichinde Virus Antigens and Their Application to Screening Hybridomas for Antibody to Pichinde Virus

A. Introduction

Acetone of other membrane-dissolving fixatives permeabilize cells to the extent that antibodies can enter the interior of the cell. Fluorescence microscopy is too time consuming and ambiguous to distinguish between antigens on the cytoplasmic membrane and antigens within the cell if the cells are permeable to antibodies. When infected cells are to be used as the targets in immunofluorescence assays for cell surface-associated viral antigens, the use of membrane-dissolving fixatives is undesirable and cells fixed with nonpermeabilizing fixative are preferred. In these studies we examined several options for producing Pichinde virus-infected target cells suitable for monoclonal antibody screening procedures specific for viral antigens expressed on the cell surface. Various fixatives were evaluated and an indirect immunofluorescence assay utilizing formaldehyde-fixed target cells was found to be a satisfactory screening procedure. However, acetone fixation was still found to offer some advantages over the other options examined, the primary advantage being that acetone-fixed target cells could be prepared in advance and stored for several months without significant deterioration. A result of these studies was the development of a two stage procedure for screening hybridomas, with both stages utilizing indirect immunofluorescence assays using fluorescein-labeled goat antibody to mouse immunoglobulin. The screening procedure developed for the first stage is a quicker, more convenient assay designed to

eliminate the majority of the inappropriate antibodies. By using acetone-fixed target cells, additional hybridomas secreting antibodies that react with Pichinde virus antigens not expressed on the cell surface are detected and, although these monoclonal antibodies may not be suitable as drug carriers, they can be used to develop a panel of monoclonal antibodies toward Pichinde virus. The more rigorous second stage, utilizing formaldehyde-fixed target cells, detects antibodies specific for Pichinde virus antigens expressed on the cell surface. These latter antibodies that recognize virus-specific cell-surface antigens may be useful in drug-targeting experiments.

B. Materials and Methods

Cells. The Vero 76 line of African green monkey kidney cells was obtained from the ATCC and cultured in MEM (GIBCO) containing 9% FBS (HyClone). For immunofluorescence assays, cells were seeded at 6 x 10^4 cells/well in 96-well polystyrene plastic cell culture plates (Corning 25861-96, Corning, NY).

Virus. The An4763 strain of Pichinde virus (obtained from Dr. Joseph D. Gangemi, University of South Carolina School of Medicine, Columbia, SC) and the CoAn3739 strain of Pichinde virus (obtained from the ATCC) were used in these studies.

Virus Assays and Antibody Assays. Pichinde virus and anti-Pichinde virus antibodies were assayed by an indirect immunofluorescence assay as described in Section X of this report.

Antisera. Antisera used in these studies were: as a positive control and for the purpose of assay development, hyperimmune anti-Pichinde virus sera prepared in adult C57Bl/6 mice as described in Section IX of this report, and as the second antibody in the indirect immunofluorescence assay, affinity-purified goat anti-mouse IgG fluorescein conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Pichinde Virus Infection of Vero Cells. Vero 76 cells growing in 96-well cell culture plates were infected with Pichinde virus as described in Section X of this report.

Preparation and Storage of Acetone-Fixed Target Cells for Antibody Screening. For the initial screening assay, Pichinde virus-infected Vero cells in 96-well cell culture plates were fixed with acetone 22 hours post-inoculation. These acetone-fixed cells were stored dry at -15°C for subsequent hybridoma screening. Cells stained after 4 months of storage still gave excellent results.

Preparation of Fixatives. Formaldehyde fixatives were prepared fresh from paraformaldehyde to avoid the methanol that is commonly present in commercially available formaldehyde solutions. One gram of paraformaldehyde (Sigma) was placed in 12.5 ml of phosphate-buffered saline (PBS) (8% formaldehyde) and then the paraformaldehyde was dissolved at 60°C by dropwise addition of 0.1 N NaOH until the solution cleared (the pH was then adjusted to 7.2). From the 8% solution, the less concentrated solutions were made by diluting in PBS. Glutaraldhyde

solutions were made by diluting highly purified glutaraldehyde (Grade 1 glutaraldehyde from Sigma) in PBS.

Fixation. Acetone fixation was performed as described in Section X of this report. Essentially, cells were exposed to cold 80% acetone for five minutes and then air-dried. For formaldehyde and glutaraldehyde fixations, the fixed cells were not allowed to dry prior to reaction with the primary antisera. This was accomplished by adding PBS to the fixed cells as soon as the fixative was removed. The cold fixative (4°C) was placed on the cells for five minutes. All fixatives were diluted in PBS. Fixed cell sheets were rinsed twice with PBS and then the primary antisera was added without letting the cells dry. In some instances, glutaraldehyde-fixed cells were treated for 10 minutes with 0.1 M glycine in PBS (to block unreacted aldehyde groups) prior to addition of the primary antisera.

Immunofluorescent Staining of Pichinde Virus-Infected Cells. Immunofluorescent staining was by the indirect procedure as described in Section X of this report.

Viewing. Samples were viewed at 160X with a microscope equipped with epifluorescence optics.

C. Results and Discussion

Preparation of Acetone-Fixed Target Cells for Antibody Screening. Vero cells in 96-well cell culture plates were inoculated with the An4763

strain of Pichinde virus and then individual plates were fixed with acetone at 15, 22, and 27 hours post-inoculation. The cultures were then stained for Pichinde virus using an indirect immunofluorescence procedure. When the infection was terminated at 15 hours post-inoculation, there was very little viral antigen present in the infected cells. When the infection was stopped at either 22 or 27 hours post-inoculation, there were approximately 5 fluorescing cells per 160X-microscope field with no increase in immunofluorescent cell count associated with the extra 5 hours of infection. The immunofluorescent staining intensity appeared to be only slightly greater in the culture infected for 27 hours as opposed to 22 hours. However, both of these incubation times resulted in much greater immunofluorescent staining intensities than did the 15-hour incubation. Previously we observed that infection periods of 36 or more hours resulted in secondary infections that tended to make the contrast between infected and noninfected cells less distinct. When acetone-fixed cells that had been stored four months at $-15^{o}C$ were compared with freshly prepared acetone-fixed cells, the immunofluorescent staining intensity was essentially identical for the two.

Evaluation of Fixatives for the Nonpermeabilizing Fixation of Pichinde Virus-Infected Vero Cells. Two commonly used fixatives that do not permeabilize the cytoplasmic membrane, formaldehyde (HCHO) and glutaraldehyde [$OHC(CH_2)_3CHO$] were examined in these experiments.

Preparation of Formaldehyde Solutions from Paraformaldehyde. Formalin is a 37 to 40% solution of formaldehyde. Commercially available formaldehyde solutions frequently contain 10 to 15% methanol. These methanol-

containing solutions are not suitable fixatives if permeabilization of the cell membrane is to be avoided. For these studies, formaldehyde solutions were prepared fresh from paraformaldehyde. Glutaraldehyde fixation resulted in consistently disappointing results; as glutaraldehyde-fixed cells were examined, it became apparent that these cells had a tendency to nonspecificaly bind antibodies. In an attempt to counteract this phenomenon, 0.1 M glycine in PBS was added to the fixed cells prior to addition of antibody in order to saturate the free aldehyde residues. The fixative conditions examined are listed in Table XI-1. The evaluations of these fixatives are described in the following results sections.

Effectiveness of Fixatives at Fixing Cells to Plastic Cell Culture Plates. To determine which conditions fixed cells sufficiently to the plastic, cell sheets were examined at 40X magnification with an inverted microscope after each step of the immunofluorescent staining procedure. The following fixatives firmly fixed cells to plastic: 80% acetone, 0.25% glutaraldehyde, 8% formaldehyde, 4% formaldehyde, and 2% formaldehyde; 0.025% glutaraldehyde and 1% formaldehyde fixation were much less effective, resulting in loss of 70 to 90% of the cells through the rinsings and stainings. Concentrations of formaldehyde below 1% were completely ineffective at attaching cels to the 96-well cell culture plates.

Autofluorescence. There was only the slightest amount of autofluorescence associated with the acetone or formaldehyde-fixed cells. The 0.25% glutaraldehyde caused a high level of autofluorescence as

demonstrated by bright yellow fluorescence even when no fluorescent conjugate was placed on the cells. The autofluorescence in the 0.025% glutaraldehyde-fixed cells was also high but it was much lower than in the 0.25% glutaraldehyde-fixed cells. Although it was possible to detect the Pichinde virus-infected cells against the autofluorescing background in the 0.25% glutaraldehyde-fixed cells, most of the cells had come off these wells since 0.025% glutaraldehyde fixation did not firmly attach the cells to the plastic.

Nonspecific Staining. The nonspecific staining in the acetone-fixed cells was just barely visible; actually, it was desirable since it allowed the observer to focus the microscope on the cell sheet in the absence of infected cells. There was less nonspecific staining in the formaldehyde-fixed cells and it was difficult to determine if the cell sheet was in focus unless there were some fluorescing cells present--that is, there was virtually no background staining. For this reason, in the hybridoma screening assay it was frequently preferable not to rinse the fluorescein-labeled anti-mouse antibody from the cells, but, rather, to just pour it off so that there was a faint background on which to focus. Initially there appeared to be a problem of nonspecific staining in the glutaraldehyde-fixed cells, but the greater portion of this staining turned out to be autofluorescence. Post-treatment of glutaraldehyde-fixed cells with 0.1 M glycine did not significantly reduce the background, but that may have been because the autofluorescing background was so high that nonspecific staining was minor in comparison.

Specific Staining. The acetone-fixed cells gave the most intense specific staining, so they were very easy to see. The 8% formaldehyde, 4%

formaldehyde, 2% formaldehyde, and 1% formaldehyde-fixed cells all resulted in very specific staining, although not as intense as the acetone-fixed cells. The 4% and 2% formaldehyde-fixed cells seemed to give the best contrast for the specific staining. The lower the concentrations of formaldehyde failed to firmly fix the cells to the plastic. In the 0.025% glutaraldehyde-fixed cultures, fluorescent cells were far less distinct than in the formaldehyde-fixed cultures. The intense autofluorescence associated with the 0.25% glutaraldehyde-fixed cultures rendered any specific fluorescence undetectable. The specific staining pattern in the formaldehyde-fixed cultures was membrane-associated, whereas that in the cells fixed with acetone or with formaldehyde followed by acetone was distributed throughout the cytoplasm and over the cytoplasmic membrane.

Effect of Aldehyde Fixation Followed by Acetone Fixation on the Immunofluorescent Staining of Cells. The cultures fixed with 4% formaldehyde and then fixed with acetone prior to staining displayed more intensely fluorescing cells than did cultures fixed with formaldehyde alone and had about the same level of fluorescence as acetone-fixed cells. So it appears that formaldehyde fixation does not significantly damage the antigens and also it seems that acetone fixation exposes additional Pichinde virus antigens in the cells. The glutaraldehyde-fixed cells that were subsequently acetone-fixed also had readily visible fluorescing cells although the contrast and clarity against the objectionable autofluorescing background were greatly reduced.

Effect of Drying Formaldehyde-Fixed Cells prior to Immunofluorescent Staining. Our initial protocol used formaldehyde-fixed cells that were allowed to dry prior to staining (the fixed cells were allowed to dry, in an attempt to more firmly attach the cells to the plates). With that protocol we failed to detect significant differences in staining patterns between acetone-fixed and formaldehyde-fixed cells. Apparently there was something in the procedure that resulted in the permeabilization of the formaldehyde-fixed cells. One possibility was that the formaldehyde-fixed cells suffered extensive membrane damage when allowed to dry after fixation. An experiment was conducted wherein Pichinde virus-infected Vero cells were fixed with 4% formaldehyde and then processed for immunofluorescent staining either with or without being allowed to dry. Acetone-fixed cells were included for comparison. The results are shown in Table XI-2. The cells that were fixed with 4% formaldehyde and then allowed to dry prior to reaction with the antibody toward Pichinde virus, became permeable to antibody and thus cytoplasmic antigens were stained. The cells that were fixed with 4% formaldehyde and not allowed to dry prior to reaction with the primary antibody were predominantly impermeable to the antibody and cytoplasmic antigens were generally not stained; only membrane associated antigens reacted with the antibody toward Pichinde virus.

Effect of Storing Formaldehyde-Fixed Cells prior to Immunofluorescent Staining. Can Pichinde virus-infected cell cultures be prepared ahead of time, fixed with formaldehyde, and then stored for later use? Apparently not. When formaldehyde-fixed cells that had been stored in PBS for 24 hours at 4°C were used as target cells for immunofluorescent staining, they appeared to have lost their

impermeability to immunoglobulin. The staining pattern became more like that of the acetone-fixed cells, but much less intense.

Two-Stage Procedure for Screening Hybridomas for Antibody to Pichinde Virus. As a result of the observations described in this section and in Section X, a two-stage procedure for screening hybridomas was developed (Table XI-3). Both stages utilize indirect immunofluorescence assays using fluorescein-labeled goat antibody to mouse IgG. The first stage is a quicker, more convenient assay that eliminates the majority of the inappropriate hybridomas. The more rigorous second stage detects antibodies specific for cell surface-associated Pichinde virus antigens, does not detect antibodies reacting with Pichinde virus antigens not expressed on the cell surface, and indicates those antibodies that react with Vero cell antigens. In the first stage, all wells showing hybridoma growth are screened for anti-Pichinde virus antibody by immunofluorescence assay using 96-well plates of acetone-fixed Pichinde virus-infected Vero cells as targets. The initial screening step detects anti-Pichinde virus antibodies specific for all Pichinde virus antigens. In the second step, those hybridomas that are positive in the initial screening are screened for antibody to Pichinde virus antigens expressed on the cell surface by using formaldehyde-fixed cells as the target cells. To avoid selection of antibodies that react with Vero cell antigens, the 96-well microtiter plates used in the secondary screening are set up with alternating rows of wells containing Pichinde virus-infected and noninfected Vero cells. Each sample is tested against infected and noninfected Vero cells.

Using the two stage screening procedure as opposed to screening with either acetone-fixed or paraformaldehyde-fixed target cells alone has some advantages:

1) The acetone-fixed target cells for the primary stage may be prepared ahead of time and stored for several months, whereas the formaldehyde-fixed target cells must be prepared on the day that they are to be used since they cannot be stored and still retain membrane integrity.

2) The disadvantages of acetone fixation—permeabilization of the cell and lack of specificity for Pichinde virus antigens on the cell surface—are avoided by using acetone-fixed cells, because hybridomas secreting antibody toward cell surface or cytoplasmic Pichinde virus antigens are detected and thus a panel of monoclonal antibodies covering the spectrum of Pichinde virus epitopes may eventually be constructed.

3) The secondary screening stage allows the specific antibodies that will be used in drug delivery to be selected.

4) Screening with the acetone-fixed cells is more convenient since a supply of target cell plates can be maintained ready for an assay, any number of wells can be used on a plate, and at the end of the assay the plates can be stored for later use of the remaining unused wells; this facilitates frequent screening of small numbers of samples.

5) The inclusion of the noninfected cell controls in the secondary screening stage and not in the primary stage reduces the number of wells by half in the initial stage of screening, when the number of samples is the greatest.

D. Conclusions

Two characteristics of glutaraldehyde rendered it an undesirable fixative in the indirect fluorescent antibody assay. First, there was a nonspecific attachment of test sera to glutaraldehyde-fixed cells, presumably a result of the bifunctional nature of glutaraldehyde. Second, glutaraldehyde-fixed cells were found to autofluoresce at the excitation wavelength of fluorescein.

Acetone-fixed Pichinde virus-infected Vero cells may be prepared in advance and stored at $-15^{o}C$ prior to serving as target cells in immunofluorescence assays for antibodies toward Pichinde virus. These acetone-fixed cells display intense specific staining with very little nonspecific staining or autofluorescence. However, acetone-fixed cells are not suitable for distinguishing cytoplasmic antigens from cell surface antigens.

A 4% solution of formaldehyde is a suitable fixative for immunofluorescence assays for antibodies toward cell surface-associated Pichinde virus antigens. As long as they are not allowed to dry, formaldehyde-fixed cells retain a permeability barrier to antibodies.

However, formaldehyde-fixed cells could not be stored for more than a few hours and still retain the permeability barrier. The formaldehyde-fixed cells adhere reasonably well to the plastic cell culture plate thus facilitating the series of washes and reagent changes inherent in the indirect immunofluorescence assay.

The combined advantages and disadvantages of acetone-fixed target cells and formaldehyde-fixed target cells can be balanced in a two-stage hybridoma screening procedure for antibodies toward Pichinde virus. The first stage takes advantage of the simplicity and convenience of acetone-fixed cells. While the second stage, which is applied to a more select sample population, utilizes the increased specificity resulting from the use of formaldehyde-fixed cells and the incorporation of cell controls.

Table XI-1. Fixation Conditions Evaluated for Preparing Pichinde Virus-Infected Vero Cells for Immunofluorescence Assay.

80% acetone
0.25% glutaraldehyde
0.025% glutaraldehyde
0.025% glutaraldehyde followed by 80% acetone
0.25% glutaraldehyde followed by 80% acetone
0.25% glutaraldehyde followed by 0.1 M glycine
0.025% glutaraldehyde followed by 0.1 M glycine
8% formaldehyde
4% formaldehyde
2% formaldehyde
1% formaldehyde
0.5% formaldehyde
0.25% formaldehyde
4% formaldehyde followed by 80% acetone

Table XI-2. Comparison of Immunofluorescent Staining in Formaldehyde-Fixed Pichinde Virus-Infected Cells That Had Been either Dried prior to Reaction with the Anti-Pichinde Virus Antibody or Maintained in Physiological Saline.

Fixative	Observations
Acetone	Approximately 10 fluorescing cells per field; staining was intense and evenly distributed throughout the cytoplasm with some speckles and rarely some blobs of stained antigen in cytoplasm.
4% formaldehyde (cells dried)	The pattern was similar to that in the acetone-fixed cells, but the fluorescence was not so intense; there appeared to be more membrane associated fluorescence. There were about the same number of fluorescing cells as in the acetone-fixed cultures.
4% formaldehyde (cells kept moist)	The pattern of fluorescence was very different from that seen in either the acetone-fixed or the formaldehyde-fixed cells that were allowed to dry. There were 50 to 75% fewer fluorescing cells per field than were observed with either of the other fixation procedures. Those cells that were fluorescing displayed 4 to 10 brightly fluorescing, well-defined spots on their cytoplasmic membranes and some finer speckles on the membrane. The evenly distributed cytoplasmic staining as seen in the acetone-fixed cells was not observed.

Table XI-3. Two-Stage Protocol for Immunofluorescence Screening of Hybridoma Supernatant Media for Antibodies toward Pichinde Virus.

1. Prepare acetone-fixed Pichinde virus-infected Vero cells in 96-well plates and store dry at $-15^{o}C$ until needed for screening.
2. Add 100 µl of hybridoma supernatant media sample per well to the acetone-fixed cells. Assay supernatant media undiluted and diluted 1:100 in PBS. Incubate at $37^{o}C$ for 1 hour.
3. Remove samples and gently rinse cells three times with PBS, 250 µl per well.
4. Add 100 µl of affinity-purified, fluorescein-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals), diluted 1:500 in PBS, and incubated at $37^{o}C$ for 1 hour.
5. Remove fluorescein-conjugated second antibody. If needed, rinse cells once with distilled water. Add a few drops of elvanol mounting media to each stained well and examine at 160X using epifluorescence microscopy. A positive sample results in brightly fluorescing infected cells contrasted against nonfluorescing uninfected cells in the same field.
6. Those hybridoma supernatant media that test positive in the initial stage are then assayed in the second stage for activity toward Pichinde virus antigens expressed on the cell surface.
7. Formaldehyde-fixed target cells in 96-well plates with alternating rows of wells containing Pichinde virus-infected and noninfected Vero cells are prepared on the day of the assay. To avoid selection of antibodies that react with Vero cell antigens, each sample is tested against infected and noninfected Vero cells.

8. Add 100 μl of hybridoma supernatant media samples per well to Pichinde virus-infected and cell control wells. Assay supernatant media undiluted (if first stage indicated a very high antibody concentration, then supernatant samples should be diluted in PBS). Incubate at 37°C for 1 hour.

9. Remove samples and gently rinse cells three times with PBS, 250 μl per well.

10. Add 100 μl of affinity-purified, fluorescein-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals), diluted 1:500 in PBS, and incubate at 37°C for 1 hour.

11. Remove fluorescein-conjugated second antibody. If needed, rinse cells once with distilled water. Add a drop of elvanol mounting media to each stained well and examine the virus-infected wells at 160X using epifluorescence microscopy. A positive sample results in brightly fluorescing specks and patches on the cytoplasmic membrane of infected cells. When a positive sample is found, examine the corresponding cell controls.

XII. Culturing the FOX-NY Cell Line

A. Introduction

The FOX-NY cell line is a myeloma cell line that has recently been recognized as a desirable cell line for hybridoma derivations. The advantages of using the FOX-NY cell line are described in the original reference to the technique by Taggart and Samloff.[1] The FOX-NY cell line is deficient in the enzyme adenosine phosphoribosyltransferase (APRT), which is required for growth under APRT$^+$ selection, that is in AAT selection media (7.5×10^{-5} M adenine, 8×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine). The FOX-NY cell line does not produce immunoglobulin heavy or light chains. Mice of the RBF/DnJ strain are characterized by a mutation resulting in a Robertsonian 8.12 translocation, which places portions of chromosomes 8 and 12 on a single 8.12 translocation chromosome. Because the gene coding for APRT is on chromosome 8 and the heavy chain immunoglobulin locus is on chromosome 12, the enzyme marker sensitive to the selection procedure is genetically linked to the ability to produce immunoglobulin. The overall outcome of this linkage is the generation of stable antibody-producing hybridomas.

Having no previous experience with the FOX-NY cell line, we had to first determine the growth characteristics of the cell line in various media that were to be used for the maintenance, fusion, cloning, and freezing of the line and the derived hybridomas. We

examined the effects of several commercially available media supplements as well as antibiotics on the growth of the FOX-NY cells in order to detect any deficiencies in the recommended basal media and then to appropriately supplement the growth media to be used for hybridoma production. It had been reported to us that the FOX-NY cell line could be cloned in the absence of feeder cells--a strong advantage for the FOX-NY line over other myeloma cell lines because feeder cells are a significant source of contamination, which may result in the loss of valuable hybridoma cell lines. Furthermore, the presence of feeder cells in a cloning plate would increase the difficulty of detecting multiple clones of hybridoma cells in a single well, and, thus, would decrease the reliability of the cloning procedure. We examined the cloning of FOX-NY cells in the absence of feeder cells.

Prior to cloning viable hybridoma cells, the mixture of spleen cells, myeloma cells and hybrids of the two are screened in selective media. This procedure should result in the selection of only hybridoma cells. The selection of hybrids will require that the hybridoma cells be grown in RPMI-1640 medium supplemented with aminopterin, adenine, and thymidine (AAT) and then cloned in AT (AAT less the aminopterin). The FOX-NY cells should die in RPMI-1640 supplemented with AAT. Prior to performing fusion procedures, we examined the effects of AT and AAT on FOX-NY cells to determine if the FOX-NY cell line responded as predicted to these reagents.

B. Materials and Methods

Cells. Cells used in this study were the FOX-NY murine myeloma cell line (HyClone Labs). Cells were passaged in polystyrene disposable cell culture flasks. The FOX-NY cells were grown in RPMI-1640 medium (American Biorganics, North Tonawanda, NY) containing 15% FBS. Cells were vigorous and in the logarithmic-phase of growth at the time of freezing.

Cell Counting and Viability Determinations. Cell counting was performed with a hemacytometer. For viability determinations, 0.04% trypan blue (Sigma Chemical) was included in serum-free cell suspensions.

Media Supplements. A study was conducted to determine if supplementation of the RPMI-1640 media with commonly used supplements would lead to improved FOX-NY cell growth. Media supplements used were: glutamine at 2 mM, essential amino acids (Sigma 50X), nonessential amino acids (Sigma 100X), vitamin solution (Sigma 100X), and sodium pyruvate (Sigma 100X); all were purchased from Sigma Chemical Co. as sterile cell culture-tested reagents and used at the manufacturers' prescribed concentrations. The effect of these supplements on FOX-NY cell growth was followed by cell counting using a hemacytometer. Similarly, the effect of antibiotics gentamicin (50 μg/ml) and a combination of penicillin (100 Units/ml)-streptomycin sulfate (100 μg/ml) on FOX-NY cell growth was examined (antibiotics also purchased

from Sigma).

Effects of Adenine, Aminopterin, and Thymidine on Growth of FOX-NY Cells. To determine the effect of RPMI-1640 containing 7.5×10^{-5} M adenine and 1.6×10^{-5} M thymidine (RPMI-1640 with AT) on the growth of FOX-NY cells, the cloning efficiency of FOX-NY cells was compared in media with and without AT supplementation. FOX-NY cells from a culture growing in log-phase were diluted in RPMI-1640 medium (containing 15% FBS, 0.01 M HEPES, 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate) to a density of 10 cells/ml. Then 0.10 ml of this cell suspension was added to each well of four 96-well plates; the wells in two of the plates contained RPMI-1640 media and wells in the other two contained RPMI-1640 plus 2XAT media (0.10 ml of media in each well, so the total volume was 0.20 ml). The plates were then covered with Saran Wrap and incubated at 37°C until the clones were counted three days later. The effect of the AAT selection medium (RPMI-1640 with AT plus 8×10^{-7} M aminopterin) on FOX-NY cells was examined by diluting FOX-NY cells into AAT medium, seeding a 96-well cell culture plate at 1×10^{-4} cells/well in AAT medium, and then monitoring the cell densities by gross examination with an inverted microscope and by cell counting with a hemacytometer.

Conditioned Media. Conditioned medium was produced by culturing FOX-NY cells (2×10^{5} cells/ml to 1×10^{6} cells/ml) in RPMI-1640 medium containing 15% FBS for 48 to 72 hours and then removing the supernatant medium and filter-sterilizing it.

C. Results and Discussion

Effects of Various Media Supplements on the Growth of FOX-NY Myeloma Cells. FOX-NY cells, in the media tested, were distributed at 2.0×10^5 cells per ml (1.5 ml per well) into 24-well cell culture plates. To do this, the cells were counted and then suspended at 3.0×10^6 cells/ml in RPMI-1640 without supplements and distributed at 0.1 ml/well. Then 1.4-ml aliquots of each of the test media were added to four replicate wells. Cell counts were performed three days post-seeding. As shown in Table XII-1 none of the media supplements increased the rate of population growth.

Effect of Adenine and Thymidine on Growth of FOX-NY Cells. Because at the time of seeding it was very time consuming and not very reliable to scan the cloning plates and pick out those wells containing a single cell, this procedure for detecting single colony wells was abandoned as impractical. However, at three to five days post-seeding, scanning the wells for the number of clones in a well was relatively easy.

When the cloning plates were scanned at 40X three days after seeding, it was easy to spot colonies of cells (single cells were much more difficult to spot quickly). After spotting a colony, the magnification could be increased to confirm that cells were present. Three days post-seeding, there were FOX-NY cell colonies in 21% of the wells

in the plates containing RPMI-1640 and colonies in 32% of the wells of the plates containing RPMI-1640 with adenine and thymidine. The number of cells per well ranged from two to 31, with most colonies consisting of six to twelve cells. A single cell was not counted as a colony.

Effect of Aminopterin on Growth of FOX-NY Cells. The cells in the AAT plate were examined at 24 hours post-seeding. There was considerable debris indicating cell lysis and degradation. A viable cell count gave 5,000 cells/ml with 66% still viable. Originally, the wells were seeded at 50,000 cells/ml and greater than 98% were viable. So, in 24 hours the viable cell density had dropped by over 95%. At four days post-seeding, few of the originally seeded FOX-NY cells were recognizable as cells and fewer than 0.4% of the originally seeded cells were still viable after four days in AAT media.

Cloning FOX-NY Cells in Conditioned Media. Cloning experiments similar to that described above for the effect of AT are being conducted using conditioned medium. In the initial trials, conditioned RPMI-1640 medium was far superior to regular RPMI-1640 with respect to the proportion of clones that progress beyond the six to twelve-cell stage. With RPMI-1640, more than 80% of the clones died out before reaching a size sufficient to cover half of the well in a 96-well culture plate. However, when FOX-NY cells were cloned in conditioned media, more than 50% of the clones that reached the six to twelve-cell stage went on to produce clones that covered at least half of the

well. Further studies are being performed to determine the feasibility of cloning FOX-NY cell-derived hybridomas in conditioned media without feeder cells.

D. Conclusions

Media supplements are sometimes added to media to improve the growth of fastidious cells or to correct media deficiencies. We found the medium RPMI-1640 containing 15% FBS to be satisfactory for culturing FOX-NY cells and saw no increase in growth rate upon supplementation of the medium with glutamine, essential amino acids, nonessential amino acids, vitamin solution, sodium pyruvate, or a combination of all five of these supplements. Although RPMI-1640 medium was suitable for the general culture of FOX-NY cells, the efficient cloning of these cells was better accomplished with conditioned media prepared from RPMI-1640 medium in FOX-NY cells that had been cultured. The antibiotic combination of penicillin (100 Units/ml)-streptomycin (100 μg/ml) was not significantly toxic to FOX-NY cells.

Within four days, AAT selection medium containing aminopterin killed over 95% of the nonfused FOX-NY myeloma cells that would be present in a fusion mixture. The adenine and thymidine added to the selection mixture to supplement the salvage pathway mediated by the enzyme adenosine phosphoribosyltransferase, which is present in hybridoma cells but not FOX-NY cells, were not toxic to the myeloma cells.

E. Literature Cited

1. Taggart, R. T. and Samloff, I. M. 1983. Stable antibody-producing murine hybridomas. Science, 219: 1228-1230.

Table XII-1. Effects of Various Media Supplements on the Growth of FOX-NY Myeloma Cells.

Media	Cell Density[a] after Three Days in Media (cells/ml × 10^5)
RPMI-1640	15.0 ± 0.4
RPMI-1640 + Pen-Strep	12.9 ± 1.0
RPMI-1640 + glutamine	11.4 ± 0.5
RPMI-1640 + essential amino acids	11.6 ± 1.0
RPMI-1640 + nonessential amino acids	13.2 ± 0.4
RPMI-1640 + vitamin solution	12.6 ± 0.8
RPMI-1640 + sodium pyruvate	11.7 ± 1.9
RPMI-1640 + all five supplements	11.0 ± 1.2
RPMI-1640 + all five supplements + Pen-Strep	13.7 ± 0.7

[a]Cells were seeded at 2 × 10^5 cells/ml in the indicated media, using four replicate wells for each medium formulation. Cell densities were determined three days post-seeding.

XIII. Development of a Procedure for Freezing Cells

A. Introduction

Reliable procedures for freezing and thawing of cells are essential for hybridoma production. The myeloma and hybridoma lines are frozen for storage and distribution. Valuable hybridoma cell lines must be safeguarded against loss by being grown, frozen, and then thawed when needed. As the work load increases during the cloning and expansion phases of hybridoma production, many hybridomas must be frozen for later characterization. Cooling equipment with programmable, controlled cooling rates, generally around 1°C/minute, is ideal for freezing cells. To control the cooling rate when freezing cells, we use insulated containers to slow the rate of cooling when cell suspensions are placed in an -80°C freezer. The procedure has been satisfactory for several cell lines routinely used in our laboratories, but we had not previously applied it to myeloma or hybidoma cell lines. The procedure was adapted to the FOX-NY cells and their derived hybridoma cell lines by substituting the appropriate media. We then tested the procedure on the FOX-NY myeloma cell line.

B. Materials and Methods

Cells. The cells used in this study were the FOX-NY murine myeloma cell line (HyClone Labs, Logan, UT). The cells were passaged in polystyrene disposable cell culture flasks. The FOX-NY cells were grown in RPMI-1640 medium (American Biorganics, North Tonawanda, NY) containing

15% FBS (HyClone Labs, Logan, UT). Cells were vigorous and in the logarithmic phase of growth at the time of freezing.

Freezing Media. RPMI-1640 medium with 40% FBS and RPMI-1640 medium with 20% dimethyl sulfoxide (Sigma Chemical, St. Louis, MO), both sterile-filtered, were used.

Freezing Procedure. Cells were pelleted by centrifugation, and the cell pellet was resuspended in RPMI-1640 without FBS. The total number of cells was determined using a hemacytometer. The cells were again pelleted and suspended in cold RPMI-1640 with 40% FBS to yield between 2×10^6 and 2×10^7 cells per milliliter (at this point the volume was one-half the final volume). From this step on, the cells and all media were held at 0°C to 4°C. An equal volume of cold RPMI-1640 with 20% dimethyl sulfoxida was added dropwise to the cell suspension. The cells were dispensed (1 ml/tube) into 2-ml polypropylene cryotubes with silicone gasket (Nunc, Danmark) placed on ice. The cryotubes were placed in a styrofoam test-tube holdar (50-tube package of 15-ml disposable centrifuga tubes, Item 25319, Corning Glass Works, Corning, NY), which was placed in a -80°C freazer. Aftar 12 to 24 hours the cryotubes were transferred to boxes or canes and stored in a liquid nitrogen or an ultracold (-135°C) freezer.

Recovery of Frozen Calls. The cryotubes of cells were allowed to partially thaw at 37°C; as soon as the pellet of ice was free from the walls of the tube, the contents were poured into centrifuge tubes containing 10 ml of RPMI-1640 with 40% FBS. The cells were pelleted and

the medium was removed. The cells were gently resuspended in 10 ml of RPMI-1640 with 15% FBS, seeded into a T25 flask, and incubated at 37°C.

Cell Counting and Viability Determinations. Cell counting was performed with a hemacytometer. For viability determinations, 0.04% trypan blue (Sigma) was included in the cell suspensions.

C. Results and Discussion

A culture of FOX-NY cells was frozen by the outlined procedure and individual cryotubes stored at -80°C were thawed at 2 days and 30 days after freezing. The outgrowth of these thawed cultures was followed by performing viable cell counts. The results are summarized in Tables XIII-1 and XIII-2.

From the data in Table XIII-1, the effect of the freezing procedure independent of the effect of prolonged storage at -80°C was a loss of approximately 20% of the viable cells, but the remaining viable cells were capable of quickly resuming vigorous growth. The data in Table XIII-2 indicate an additional loss of 10-20% of the viable cells as a result of storage for 30 days at -80°C.

D. Conclusions

The cell-freezing and -recovery procedures described above appear to be satisfactory for the FOX-NY myeloma cell line. For short-term storage (1 to 3 months), -80°C may be sufficiently cold; however, for longer storage it appears that lower temperatures are required. Our frozen

cells are currently being stored at both -80°C and in the vapor phase of a liquid nitrogen freezer.

Table XIII-1. Outgrowth of FOX-NY Cells Frozen for Two Days.

Time Post-Thawing	Cell Count	Viability
4 hours	1.8×10^5/ml	82%
	(1.8×10^6 cells)	
2 days	3.0×10^5/ml	97%
	(3.0×10^6 cells)	
Then split 1:5 at 2 days[a]		
4 days	3.4×10^5/ml	>98%
	(1.7×10^7 cells)	

[a] Diluted 1:5 with growth medium.

Table XIII-2. Outgrowth of FOX-NY Cells Frozen for Thirty Days.

Time Post-Thawing	Cell Count	Viability
4 hours	1.5×10^5/ml	70%
	(1.5×10^6 cells)	
2 days	3.2×10^5/ml	80%
	(3.2×10^6 cells)	
4 days	1.2×10^6/ml	90%
	(1.2×10^7 cells)	
Then split 1:3 at 4 days[a]		
5 days	7.5×10^5/ml	98%
	(2.2×10^7 cells)	

[a] Diluted 1:3 with growth medium.

XIV. Fusion Technique for the Generation of Hybridomas

A. Introduction

The FOX-NY cell line is a murine myeloma cell line that does not produce immunoglobulin heavy or light chains and thus is desirable as a fusion partner for hybridoma production. The FOX-NY cell line is deficient in the enzyme adenosine phosphoribosyltransferase (APRT), which forms the basis for the selection procedure described in the original reference[1] to the use of FOX-NY cells for hybridoma production. FOX-NY cells may be fused via polyethylene glycol to spleen cells of hyperimmunized RBF/DnJ strain mice. These mice are characterized by a mutation resulting in a Robertsonian 8.12 translocation, which places portions of chromosomes 8 and 12 on a single 8.12 translocation chromosome. The significance of this is that the gene coding for APRT is on chromosome 8 and the heavy chain immunoglobulin locus is on chromosome 12. Thus the enzyme marker sensitive to the selection procedure is genetically linked to the ability to produce immunoglobulin with the overall outcome being the generation of stable antibody-producing hybridomas.

The objective of this study was to establish a procedure for deriving hybridomas that secrete antibody to the cell-surface expressed Pichinde virus antigens. The techniques involved in fusing a myeloma cell to a spleen cell vary widely among laboratories and in the literature. The process of hybridoma derivation is quite labor

intensive and protracted. There are many critical steps at which the hybrids may be lost as a result of either contamination or untimely feedings or manipulations. For these reasons fusion techniques need to be established and practiced. The fusion procedure described in this section was derived from fusion procedures obtained from four separate laboratories: 1) the hybridoma laboratory of Dr. Mark C. Osterling at USAMRIID, Frederick, MD; 2) the hybridoma laboratory of Dr. J. Michael Mullins at the Catholic University of America, Washington, D.C.; 3) the laboratory of Dr. Mark C. Healey, Utah State University, Logan, UT; and 4) HyClone Laboratories, Logan, UT. Each of these procedures was unique at several steps. None of the four procedures was published, but each had been proved effective. The decision as to which procedure to follow at any given stage of the hybridoma production was based on the applicability to the FOX-NY myeloma cell line, the available equipment, the seeming applicability to our objectives, the relative ease and timeliness of the manipulation, and the potential for the introduction of contamination. The procedure we are currently using has yet to be proven reliable and will undoubtedly be modified as we gain experience in hybridoma production.

B. Materials and Methods

Animals. RBF/DnJ mice obtained from Jackson Laboratories (Bar Harbor, ME) were used in these studies.

Preparation of Pichinde Virus Immunogen and Immunization of Spleen Donor Mice. Pichinde virus (strain An4763)-infected suckling RBF/DnJ mouse brain pools were prepared as 10% homogenates and used to immunize eight-week-old RBF/DnJ mice with Pichinde virus as described in Section VIII of this report.

Cells. Cells used in this study were the FOX-NY murine myeloma cell line (HyClone Laboratories, Logan, UT). Cells were vigorous and in the logarithmic phase of growth at the time of fusions.

Media. The FOX-NY cells were grown in RPMI-1640 medium (American Biorganics, North Tonawanda, NY) containing 15% fetal bovine serum, 10 mM HEPES, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. In the fusion procedure and when performing viable cell counts, the above medium without serum and HEPES (RPMI-1640-SF) was used. Serum is known to interfere with both the fusion procedure and the uptake of trypan blue by nonviable cells. Some cell culture authorities claim that HEPES may be cytotoxic when cells are permeabilized by dimethyl sulfoxide (DMSO); for that reason HEPES was omitted during the fusion procedure.

Erythrocyte-Lysing Buffer. The buffer used to keep the spleen tissues moist and to lyse erythrocytes consisted of 17 mM Tris base. 140 mM NH_4Cl, filter sterilized and adjusted to pH 7.2.

hybridoma cells was as described in Section XIII of this report. The freezing medium was 10% DMSO in RPMI-1640 containing 20% FBS.

Cell Counting and Viability Determinations. Cell counting was performed with a hemacytometer. For viability determinations, 0.04% trypan blue (Sigma Chemical Co., St. Louis, MO) was included in serum-free cell suspensions.

AAT and AT Selection Media. The AAT selection media consisted of RPMI-1640 medium containing 15% FBS, 0.01 M HEPES, 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate to which was added 8×10^{-7} M aminopterin, 7.5×10^{-5} M adenine and 1.6×10^{-5} M thymidine. The AT medium was the same as AAT except without the aminopterin.

Conditioned Media. Conditioned medium was produced from RPMI-1640 medium as described in Section XII of this report.

Cytotoxicity of the Fusion Procedure with Various Polyethylene Glycol Preparations. FOX-NY myeloma cells without spleen cells were used in an experiment designed to determine the cytotoxicity of polyethylene glycol (PEG) preparations obtained from different sources. FOX-NY cells were processed through the PEG fusion steps of the hybridization procedure (Steps 9 to 15 in Table XIV-1) and then the viability of the cells was determined. The experiment was conducted

as follows. FOX-NY cells were washed 3 times in RPMI-1640-SF medium. The myeloma cells were then resuspended in the same medium and a viable cell count was performed. Into each of eight 15-ml conical polypropylene centrifuge tubes was placed 1.0×10^6 FOX-NY cells, which were then pelleted by centrifugation at $400 \times \underline{g}$ for 5 minutes. The supernatant fluids were carefully decanted and the last drops were removed with sterile swabs. To the intact cell pellet, 1 ml of the fusion mixture containing the PEG being tested was added over a period of 60 seconds with gentle agitation and then the mixtures were incubated at 37°C for 90 minutes. Each of the four PEGs was run in duplicate. The fusion suspension was then diluted by dropwise addition of RPMI-1640-SF medium, 1.0 ml over 30 seconds, 3.0 ml over the next 30 seconds, and then 10.0 ml over 60 seconds. The suspensions were allowed to stand at room temperature for five minutes. The cells were then pelleted by centrifugation at $400 \times \underline{g}$ for five minutes. Supernatant fluids were decanted, and the cells were resuspended in 2 ml of RPMI-1640-SF medium. Viability was then determined by trypan blue staining.

Fusion Mixture. The fusion mixture for the protocol described in Table XIV-1 was prepared as follows. Five grams of PEG (M.W. 4000) from Merck was placed in a 50-ml flask, and 0.5 ml of DMSO and 5.0 ml of H_2O were added. The flask was covered with foil and placed in a larger beaker with boiling chips and water to cover the liquid in the small flask. The beaker was covered with foil and processed in an autoclave at 121°C on liquid setting for 30 minutes. The mixture was

immediately aliquoted into polystyrene tubes, 2.0 ml/tube. The fusion mixture was kept sterile and sealed with parafilm. The material was stored at room temperature in the dark.

Polyethylene Glycols Tested. Four PEG variations were tested: 1) 50% PEG 4000 from Merck plus 10% DMSO prepared as described above; 2) PEG 4000 from Merck, as above except no DMSO· 3) PEG M.W. 1500, Cat. No. 547 from Hybridoma Sciences, Atlanta, GA, which was used in the first two fusions; 4) PEG 1460, M.W. 1400-1600, from ATCC, prepared by mixing 0.3 ml PEG with 0.7 ml RPMI-1640-SF medium.

C. Results and Discussion

Cytotoxicity of the Fusion Procedure with Various Polyethylene Glycol Preparations. In our attempts to produce hybridomas, the fusion cells appeared to have lost their viability, apparently at the PEG fusion step. An experiment was designed to determine if the PEG step was a problem and if other sources of PEG might solve the problem. When different PEG preparations were used for the fusion, there was a wide range in the viability of the FOX-NY cells after the fusion (Table XIV-2). The poorest viability was obtained when the PEG from Hybridoma Sciences was used and the highest viability was obtained using a 30% solution of ATCC PEG; however, because the PEG concentration of the latter preparation was lower than that usually used, the fusion efficiency may be low. The fusion mixture composed of 50% PEG 4000 (Merck) and 10% DMSO resulted in 72% viable cells. When

DMSO, which probably stabilized cell membranes by increasing their permeability thus reducing their susceptibility to osmotic lysis, was omitted from the fusion mixture the viability dropped to 60%.

Results of Fusions. Two fusions were performed using the protocol outlined in Table XIV-1 except that the PEG from Hybridoma Sciences was used as the fusogen. No viable hybridomas were derived from either of these fusions. A third fusion using the PEG from Merck as described in the protocol was performed and a few viable hybridomas resulting from that fusion are currently being expanded. These hybridomas have not reached a cell density sufficient for antibody screening. The resultant hybridoma cells were all very slow growers, reaching maximum clone sizes of only a few hundred cells by 14 days post-fusion. Since most of the clones were dying before they reached a size sufficient for antibody screening, media toxicity was suspected. Perhaps the remnants of cells killed by the selection media were contributing to this toxicity in the absence of macrophages. Fusion wells containing viable hybridomas were cloned to 96-well plates in an attempt to remove viable hybrids from the apparently toxic environment of the fusion wells. Currently, at five days post-cloning there are many apparently healthy clones at the four- to sixteen-cell stage.

D. Conclusions

The cytotoxicity of the PEG from Hybridoma Sciences resulted in unsuccessful attempts in two fusion experiments. Based on cytotoxicity measurements, the ATCC PEG was very promising, with 92% of the FOX-NY cells still viable after being exposed to a 30% solution of this reagent. We have not yet determined if a 30% solution of PEG will yield consistent cell fusions. However, a fusion mixture consisting of 50% PEG 4000 from Merck and 10% DMSO did result in a successful fusion. The antibody specifities of the hybridomas that resulted from the latter fusion have yet to be determined. The yield of hybridomas was low, however, and the fusion protocol will be modified to include the use of macrophage feeder cells in the next fusion.

E. Literature Cited

1. Taggart, R. T. and Samloff I. M. 1983. Stable antibody-producing murine hybridomas. Science, 219: 1228-1230.

Table XIV-1. Hybridization Protocol.

To prevent contamination of the "clean" hood, the initial steps are performed on the bench.

1. Anesthesize mouse with ether.

2. Bleed mouse by collecting blood from the subclavesn artery. Set the blood aside in a centrifuge tube and later harvest the serum to be used as a positive control in immunological assays.

3. Saturate animal with 70% ethanol. Peel skin from entire peritoneal and chest area, pin skin back out of way, and again saturate animal with 70% ethanol.

4. Aseptically remove the spleen. Place spleen into serum-free media in petri dish. At this point move to "clean" hood. Wear mask and change gloves. Remove excess fat and connective tissue from spleen. Transfer spleen to #60-mesh, hat-shaped stainless steel screen inverted over a 15-ml plastic centrifuge tube in an ice bath. Always keep spleen moist. While tissue is on strainer, have a helper keep tissues moist with drops of Tris-NH_4Cl to lyse the erythrocytes. After Step 5, the total volume of cell suspension (in Tris-NH_4Cl) in the tube should be approximately 10.0 ml.

5. Mince the spleen with sterile scissors. Dissociate spleen by forcing it through the stainless steel strainer using the sterile plunger from a 3.0-ml disposable syringe and rinsing with Tris-NH_4Cl. Continue this until only connective tissues remain.

6. Spin down cells at 400 × g for five minutes.

7. Decant supernate and resuspend cells in 12 ml of RPMI-1640-SF medium. Allow any large chunks to settle (two minutes) and transfer cell suspension to a duplicate tube.

8. Top off tube and spin down cells at 400 × g for five minutes.

9. At the same time, wash the above spleen cells and the myeloma cells three times in RPMI-1640-SF medium.

10. Resuspend spleen cells and myeloma cells in 7 ml of RPMI-1640-SF medium. Count viable cells.

11. Combine spleen cells and myeloma cells in a 15-ml conical polypropylene tube in a ratio of five spleen cells to one myeloma cell. Spin down cells at 400 × g for five minutes.

12. Carefully decant the supernatant and drain the tube; remove last drops with sterile swab.

13. To the intact pellet, add 1 ml of fusion mixture over 60 seconds with gentle agitation. Incubate at 37°C for 90 seconds. (Should see clumps at this stage. Be gentle, allowing clumps to remain intact. Cells are fusing at this time.)

14. Drop by drop dilute with RPMI-1640-SF medium:

1.0 ml over 30 seconds.

3.0 ml over 30 seconds.

10.0 ml over 60 seconds.

15. Spin down cells at 400 × _g_ for five minutes. Decant supernate and resuspend cells in 15 ml of RPMI-1640-SF medium. Remove small sample for viable cell count.

16. Spin down cells at 400 × _g_ for five minutes and resuspend at 1×10^6 cells/ml in AAT.

17. Distribute 1×10^6 cells/well to fresh 24-well plates. Should get approximately five plates/spleen.

18. Incubate for seven days. Feed cells 1 ml of AAT on the seventh day. On the ninth day and every day thereafter, remove 1 ml and replace with 1 ml of AAT.

19. When colonies become visible, test supernatant.

20. Clone selected wells into 96-well plates. One well of a 24-well plate into one 96-well plate.

21. Conditioned media containing adenine and thymidine (AT), but no aminopterin, is used for the first cloning. Seed one half of the plate with a cell suspension diluted sufficiently to yield one cell/well (in 0.3 ml). The remaining wells are seeded with an average of 0.5 cells/well (in 0.3 ml). To accomplish this, dilute hybrids to 100 hybrids/30 ml in conditioned media containing AT (approximately one cell/0.3 ml), and take 10 ml of this suspension and dilute with 10 additional ml of conditioned media with AT (approximately 0.5 cell/0.3 ml). Place 0.3 ml of hybrids into each well of the 96-well plates, with the top half having one-cell/well cell dilution and the bottom half at 0.5 cell/well. Colonies will become visible to the naked eye in about seven days.

22. On Day 5, examine all wells using low power (40X) and circle lid over those wells with one clone, place an X over those wells with more than a single clone.

23. When colonies cover half of the floor of the well, remove 0.1 ml of the supernatant medium, and assay it for antibody activity.

24. Expand the selected clones by using conditioned media containing AT. Expand one well to one well of a 24-well plate, 2 ml/well. Feed the 96-well cloning plate to cover for loss of the clones during expansion.

25. When 60% confluent, expand to two or three wells of a 24-well plate using conditioned media containing AT as above. (At this point hybridomas may be cloned a second time. For the second cloning, use conditioned media without AT.)

26. When 60% confluent, expand by placing all three wells into one 25-cm^2 flask containing 10 ml of conditioned media containing AT. Again when 60% confluent transfer to a 75-cm^2 flask and add 20 ml of conditioned media containing AT.

27. When flask is 60% confluent, expand as needed using RPMI-1640-SF medium without AT.

28. Freeze cells in freezing medium at 1×10^7 cells/ml.

29. Inject 1×10^6 to 1×10^7 cells intraperitoneally (IP) into pristane-primed mice (0.5 ml of pristane IP/mouse 7 to 30 days prior to injection of hybrids). Ascites development should occur in 14 to 20 days.

30. Freeze ascites cells in freezing medium, at 1×10^7 cells/ml.

Table XIV-2. Effect[a] of Various Polyethylene Glycol Preparations on FOX-NY Cell Viability.

Conditions[b]	Total cell count	Non-viable cell count[c]	Viable cells
Prior to fusion	288	11	96%
After fusion:			
PEG 4000 from Merck, with DMSO	180	50	72%
PEG 4000 from Merck, no DMSO	200	80	60%
PEG from Hybridoma Sciences	60	60	0%
PEG from ATCC	150	12	92%

[a]FOX-NY cells were processed through a mock fusion using various PEG preparations as described in the Materials and Methods section.

[b]Sources of PEG and formulations used are described in the Materials and Methods section.

[c]The distinction between viable and nonviable cells was based on trypan blue staining.

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